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Volume 43, Supplement 1, May 1954

SYMPOSIUM

ON

EFFECTS OF RADIATION AND OTHER DELETERIOUS AGENTS ON EMBRYONIC DEVELOPMENT

PUBLISHED BY
THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY
PHILADELPHIA
1954

FOREWORD

Concerning Supplements to the

JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY

A large part of the cost of most scientific journals is borne by the subscribers. The publication of lengthy manuscripts in the JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY would require a further increase in the subscription price or, alternatively, would reduce the number of pages which could be assigned to other authors who desire to publish articles relating to the many fields of physiology encompassed by this Journal. Accordingly, in the interest of our subscribers, it is necessary to limit the number of pages made available to any author and for the presentation of material dealing with any one subject.

Despite these considerations, certain authors rightly desire to publish the results of their research in an extensive form at a cost which cannot fairly be assessed against the subscribers; certain observations and data may require such a form of publication. To satisfy these desires and needs the Editorial Board of the JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY has approved, on a trial basis, the publication of occasional Supplements to the Journal. The articles published in these Supplements must meet the usual standards of scientific merit; the authors must provide the full cost of publication.

It is hoped that such a Supplement will fulfill the requirements for unique publications and will supply scientific reports of unusual interest to our subscribers at no additional cost to them.

DETLEV W. BRONK

SYMPOSIUM

ON

EFFECTS OF RADIATION AND OTHER DELETERIOUS AGENTS ON EMBRYONIC DEVELOPMENT

GIVEN AT

RESEARCH CONFERENCE FOR BIOLOGY AND
MEDICINE OF THE ATOMIC ENERGY
COMMISSION

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INTRODUCTION

The Sixth Annual Biology Research Conference on "Effects of Radiation and other Deleterious Agents on Embryonic Development" sponsored by the Biology Division of the Oak Ridge National Laboratory in cooperation with the Division of Biology and Medicine of the Atomic Energy Commission was held in Oak Ridge in the spring of 1953. The use of deleterious agents as tools for investigating basic biological problems is, of course, well known; the increasing use of radiation in this respect is of special value. The importance of this subject in connection with the wide application of high-energy radiation for diagnostic and therapeutic purposes as well as the danger involved with employment of nuclear weapons has made such a discussion rather timely.

Free and open discussions are always encouraged in these conferences. Discussions for this conference are reproduced in part. The papers of all the main speakers are given. Since Dr. B. H. Willier made an especially important contribution in the discussion, his paper is reproduced in its entirety.

One evening of the conference was devoted to a discussion of "Human implications and results of investigations by the Atomic Bomb Casualty Commission." Since most of the papers given in the evening discussion have been published elsewhere, they are not being reproduced here. The paper by Dr. J. N. Yamazaki is given here since it has not been published previously.

Dr. Liane Brauch Russell and Dr. William L. Russell were mainly responsible for the selection of papers for this conference. The committee on Developmental Biology Division of Biology and Agriculture of the National Research Council cooperated in arranging this program. This symposium is the sixth in the series which have appeared as Supplements to this Journal.

ALEXANDER HOLLAENDER

DIFFERENTIATION AND THE REACTION OF RAT EMBRYOS TO RADIATION[1]

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ONE FIGURE

The earlier studies on the effects of ionizing radiations on developing mammalian embryos have been adequately reviewed by Warkany and Schraffenberger ('47) and Russell ('50). The present report will deal not so much with the specific effects of irradiation as with changes in the pattern of response when embryos at different stages in differentiation are exposed under standard conditions. A study of this type must be as nearly quantitative as possible, which requires that sensitive criteria be set up as a basis for evaluating the responses. Previous experiments (Wilson and Karr, '51; Wilson, Jordan, and Brent, '53; Wilson, Brent, and Jordan, '53) have shown that suitable criteria were: (1) the rate of intrauterine mortality, (2) the degree of intrauterine growth retardation, and (3) the number and types of developmental malformations induced.

To obtain quantitative data it was also necessary to attempt control of several possible sources of error that are encountered when embryos are irradiated in conjunction with whole-body or abdominal irradiation of the mother. For this purpose a method was devised whereby selected embryos could be exposed to X rays without irradiation of the mother

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[1] Based in part on work performed under contract with the United States Atomic Energy Commission at the University of Rochester Atomic Energy Project.

[2] The collaboration of Dr. John W. Karr, Dr. Robert L. Brent, and Mr. H. Charles Jordan in the experiments on which this report it based is gratefully acknowledged.

or the remaining embryos. It was possible to prevent fluctuations in the depth dosage resulting from the intervention of maternal tissues of varying thickness and density by exposing the implantation sites directly, through an abdominal incision in the mother. The possibility of affecting the embryo secondarily by some primary reaction to irradiation on the part of the mother was reduced by shielding all maternal

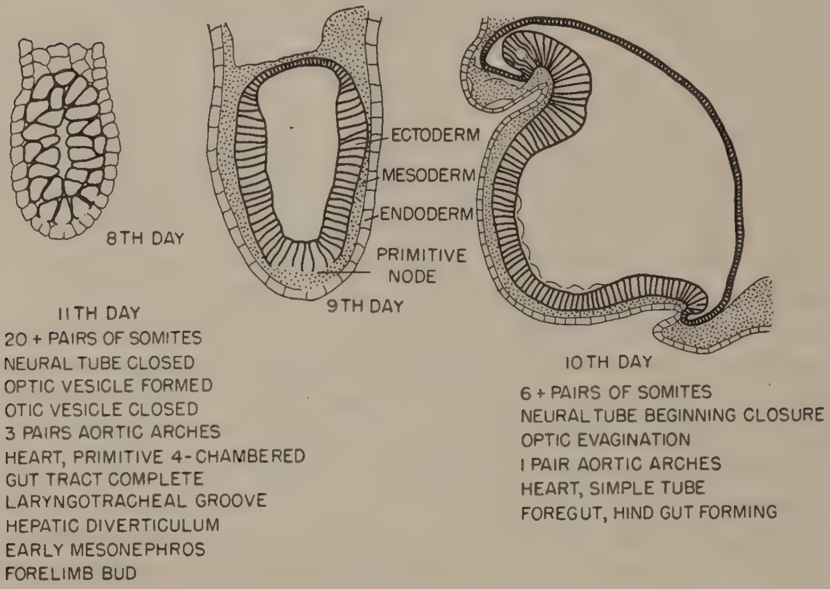

Fig. 1 Schematic representation of the stages of differentiation existing early on the eighth, ninth, tenth, and eleventh days of gestation in the rat. On the eighth day the embryo may be considered essentially an undifferentiated mass of cells. Although the surface of the embryonic cylinder is covered by primitive yolk endoderm, the significance of this layer in future development is uncertain. It is probable that many if not all cells within the embryonic mass are multipotential at this stage. On the ninth day the three primary germ layers, ectoderm, mesoderm, and endoderm, are readily identified. The polarity of the embryo has been indicated by the primitive node and streak, but organ formation has not begun. On the tenth day organ formation is under way in the nervous, cardiovascular, and digestive systems and the somatic mesoderm is beginning segmentation. By the eleventh day some degree of organ formation is in progress in almost all major systems and is moderately advanced in the cardiovascular and digestive systems. (The eleventh-day embryo is too large and complex to be represented in the scheme used here.)

tissue except the segment of uterus containing the selected implantation sites. The shielding of some embryos in each pregnancy made available nonirradiated ''litter-mate'' controls for the precise evaluation of growth and developmental changes in the exposed embryos. Exact rates of mortality were determined by comparing the number of embryos present after any postirradiation interval with the original number observed at the time of operation.

The stages of differentiation selected for such a study are of particular importance. They should represent consecutive steps in development, each characterized by easily recognized morphological features. Such a series of stepwise stages occurs between the eighth and the eleventh days of gestation in the rat (fig. 1). On the eighth day differentiation has not yet begun, at least is not apparent, for the embryo consists merely of a randomly arranged mass of blastomeres at the embryonic pole of the egg cylinder. By the ninth day the first major step in differentiation has occurred, in that the three primary germ layers have formed, but differentiation of these toward organ formation has not begun. The tenth day is characterized by the beginning of organogenesis in certain systems, particularly the nervous, cardiovascular, and digestive systems. On the eleventh day organogenesis of some degree is in progress in virtually all major systems and is moderately advanced in several. Accordingly, embryos have been exposed to X rays at these specific stages because they encompass the initiation as well as the first important steps in differentiation.

METHOD

The details of the technique by which selected embryos were exposed to direct irradiation have been discussed and illustrated elsewhere (Wilson and Karr, '51), and only the basic procedure need be repeated here. Gestation was considered to have begun at 9 A.M. of the morning on which sperm were found in the vaginal smear of female rats allowed to breed during the preceding night. The products of conception were regarded as *1 day of age* 24 hours after sperm were found. All subsequent procedures referable to the age of the embryos (exposure to X rays and termination of pregnancy) were also carried out at approximately 9:00 A.M. in order that timing might be recorded in uniform daily units.

On the eighth, ninth, tenth, or eleventh day of gestation female rats of the Wistar albino strain were anesthetized with nembutal and the ventral abdominal wall opened by a midline incision. One uterine horn was brought to the surface of the incision, and lead plates were then arranged around and underneath two to five of the implantation sites in such a manner as to shield completely all remaining embryos and all material tissues except the segment of uterus containing the unshielded sites. After exposure of the unshielded embryos, these were promptly restored to their position in the abdominal cavity. The second uterine horn was then brought to the surface of the incision and a similar number of implantation sites carried through the same manipulations, except that the latter were not irradiated. Before this horn was restored to the abdominal cavity and the incision closed, the number and position of all implantation sites in both uterine horns were noted in a diagram, to facilitate later identification of irradiated and nonirradiated embryos.

A dose of 12.5, 25, 50, 100, 200, 400, or 600 r (in air) was given at a single exposure to the unshielded embryos. A therapeutic X-ray machine calibrated with a standardized Victoreen r-meter was used. The beam was that produced by the following factors: 85 kvp, 10 ma, and 1.0 mm of aluminum added filter. Further characteristics were: half-value layer of 2.0 mm of aluminum, and 1.0 cm depth dose equivalent to the air dose. The target-object distance varied between 10 and 20 cm, and the duration of exposure never exceeded 2 minutes. The distribution of irradiated embryos with respect to dosage is presented in table 1.

After postirradiation intervals of one or more days, or early on the day of expected delivery, the mothers were killed by decapitation and both uterine horns inspected *in situ* for resorbed implantation sites. The irradiated and control embryos were identified while still *in utero* (using the diagram made at the time of exposure) and the entire uterus then removed and fixed in Bouin's fluid. After being transferred to 70% alcohol all embryos were dissected from the uterus, weighed, and examined externally under low power magnification. The irradiated embryos together with one or two of their nonirradiated siblings were serially sectioned and stained with hematoxylin and eosin for microscopic study.

TABLE 1

Distribution of irradiated embryos as to day of treatment, dosage, and number of nonirradiated siblings

DAY	NUMBER OF IRRADIATED AND CONTROL EMBRYOS IN EACH DOSAGE GROUP													
	12.5 r		25 r		50 r		100 r		200 r		400 r		600 r	
	Irr.	Con.	Irr.	Con.	Irr.	Con.	Irr.	Con.	Irr.	Con.	Irr.	Con.	Irr.	Con.
8th	13	13	27	34	28	32	27	23	15	24				
9th			17	23	49	76	178	162	43	53	10	7		
10th					30	54	62	102	56	75	19	26		
11th					4	6	18	26	40	61	25	42	14	17

OBSERVATIONS

The observations presented here on the effect of irradiation at the eighth, ninth, and tenth days of gestation are based on data published previously (Wilson and Karr, '51; Wilson, Jordan, and Brent, '53; Wilson, Brent, and Jordan, '53). In order to obtain more meaningful comparisons, however, some of the earlier data have been regrouped. Furthermore, since the emphasis in the present report is on over-all effects, the observations made on the first postirradiation day have been omitted from all groups because it has been found that typical effects frequently do not appear within so short an interval. The observations dealing with the effects of treatment on the eleventh day have not been previously published.

Mortality. The rates of intrauterine mortality after various doses of X rays given on the eighth, ninth, tenth, or eleventh day are presented in table 2. After irradiation on the ninth, tenth, or eleventh day the mortality increased as the dosage increased; but this dose-response relation was not apparent in the case of eighth-day irradiation. In the latter instance the mortality rate was not significantly increased above that of litter-mate controls until the dosage was raised to 200 r, by which dosage all exposed embryos were killed within 3 days. Thus, eighth-day embryos seem able to withstand the lethal effects of X irradiation up to a point, but beyond this, abruptly they become totally susceptible.

TABLE 2

Percentage of intrauterine mortality

DAY OF IRRADIATION	CONTROL	DOSE (r)						
		12.5	25	50	100	200	400	600
8th	9	8	0	0	13	100		
9th	8		0	11	26	74	100	
10th	14			14	23	47	100	
11th	6			0	11	35	92	100

It is also apparent from the data in table 2 that as the age of the embryo at the time of exposure advances, susceptibility to the lethal action of X rays tends to decrease. Whereas 200 r was sufficient to kill all eighth-day embryos within 3 days, 400 r was required to kill ninth- or tenth-day embryos and 600 r to kill eleventh-day embryos within a comparable period. Actually, smaller doses than those indicated were found to cause total or near-total mortality after ninth-, tenth-, or eleventh-day treatment if the postirradiation interval were lengthened to 5 days or more. Two hundred roentgen units for ninth- and tenth-day embryos and 400 r for eleventh-day embryos should perhaps be regarded as the doses that ultimately kill all exposed animals. With lower doses the rates of mortality were also lower. Again a decreasing susceptibility was noted as the age of the embryo at the time of exposure advanced beyond the ninth day.

Unlike these differences in the degree of response, shown by embryos at the ninth, tenth, and eleventh days, the eighth-day embryo exhibited a different pattern of response. Mortality did not increase in a graded fashion as the dosage increased, but suddenly became complete at 200 r.

Retardation of growth. In table 3 the deficiency in weight of irradiated embryos, as compared with their nonirradiated siblings, is expressed as percentage retardation. Since such data for animals at different postirradiation periods (from the second day to term) cannot be readily combined, data for two representative postirradiation days are presented. Despite the variability of these figures, it is clear that appropriate doses of radiation caused considerable retardation in the rate of growth of surviving embryos. The degree of retardation tended to increase with the dosage, although not in direct proportion. In most instances when retardation exceeded 30% the rate of mortality increased sharply. It is noteworthy that, unlike the observations on mortality, the relation between retardation and increased dosage was as evident after eighth-day irradiation as after treatment at any of the older ages.

TABLE 3

Retardation of growth in surviving, irradiated embryos expressed as

$$\frac{\text{mean weight of controls} - \text{mean weight of irrad.}}{\text{mean weight of controls}} = \text{percentage retardation}$$

DAY OF IRRADIATION	POSTIRRAD. DAY	DOSE (r)				
		12.5	25	50	100	200
8th	5	1	12	18	30	
8th	7	5	6	5	14	
9th	4		6	10	17	27[a]
9th	6		<1	1	17	
10th	3			0	6	23
10th	5			0	14	28[a]
11th	4				2	13
11th	5				2	9

[a] Only one animal surviving.

The dosage required to cause a comparable percentage of retardation became higher as embryos of older age were irradiated. This is suggested by the data in table 3, but is more graphically shown by comparing directly the values obtained at the same interval (5 days) after irradiation with the same dosage (100 r) on different days of gestation, as represented in table 4. Then, it may be concluded that from the eighth through the eleventh days the embryo becomes increasingly resistant to the growth-retarding influence of X rays.

TABLE 4

Percentage retardation 5 days after irradiation with 100 r

Day of irradiation	8th	9th	10th	11th
Percentage retardation	30	20 [a]	14	2

[a] See Wilson, Jordan, and Brent, '53.

TABLE 5

Types and percentage incidence of malformations produced by irradiation on different days of gestation

DAY	DOSE (r)			
	25	50	100	200
8th	None	None	None	(No survivors)
9th	Eye, 6	Eye, 72; Brain, 9; spinal cord, 3	Eye, 90; brain, 41; spinal cord, 27; heart, 20; face, 14; situs inversus, 13; arotic arcs, 10; urinary, 5	Eye, 100; brain 78; spinal cord, 67; situs inversis, 55; heart, 22; face, 11; aortic arch, 11
10th		Eye, 11	Eye, 75; urinary, 11; brain, 3	Eye, 94; feet, 33; brain, 19; urinary, 11; aortic arch, 11
11th			None	Eye, 100; urinary, 77; brain, 54; spinal cord, 31; aortic arch, 23; ear, 23; tail, 23; heart, 15; jaw, 15; feet, 7

Malformations. Embryos of different ages displayed a striking difference in sensitivity to the teratogenic influence of X rays (table 5). No malformations were produced by any dosage given to the eighth-day embryo. This is in sharp contrast to the ninth-day embryo which was found to be highly sensitive in this respect. A great variety of malformations was produced by exposure to 100 r or 200 r, and a dose as small as 25 r occasionally caused anomalous development of the eye. The tenth-day embryo was somewhat less sensitive but, nevertheless, did exhibit maldevelopment of several organs after treatment with 100 or 200 r. By the eleventh day the rat embryo was still more resistant to the teratogenic action of X rays. Malformations were not produced by any of the doses used which were less than 200 r. Undoubtedly, doses intermediate between 100 r and 200 r would have been effective, but the significant fact is that 100 r, which caused a high incidence of malformation after ninth- or tenth-day irradiation, had no effect on developmental processes at the eleventh day.

Whenever malformations occurred the eye was always the organ most frequently affected (table 5). In the lower range of effective dosage it was the only site of abnormality. After ninth-day irradiation the brain and spinal cord also were often anomalous but were less frequently affected after treatment on the tenth or eleventh day. Following tenth-day exposure the feet ranked next to the eye in order of frequency, and following eleventh-day treatment the urinary tract was the second most common site of malformation. Although the order of incidence varied, it should be noted that the same organs tended to be affected to some degree, whether exposure occurred on the ninth, tenth, or eleventh day.

Even though the same organs were often malformed by irradiation on different days, the types of malformation were not always the same. The prevalent types of ocular anomaly after either ninth- or tenth-day treatment were anophthalmia and extreme distortion of the optic cup, but eleventh-day treatment most often caused coloboma (persistent choroidal fissure). The brain was differently affected by irradiation on all 3 days: on the ninth day, an array of brain defects consequent to fusion of the brain wall with the ectoderm; on the tenth day, hypoplasia and inequalities of growth in various parts of the forebrain; and on the eleventh day, eversion of the choroid plexus and other minor distortions. The kidneys were affected by agenesis of one or both organs after ninth-day exposure, by fusion (horseshoe kidney) after tenth-day exposure, and by both types after eleventh-day exposure. Situs inversus, on the other hand, occurred only after irradiation on the ninth day, at which age it was induced with considerable frequency. The feet were often malformed after treatment on the tenth day but not at all by that on the ninth day and only occasionally by that on the eleventh day. It is evident that the same organ may remain susceptible to the teratogenic action of irradiation for several days, but the specific type of defect produced is likely to change radically with increasing embryonic age at the time of irradiation.

COMMENT

The foregoing observations have shown that the capacity of the rat embryo to react to irradiation undergoes a qualitative change between the eighth and the ninth days of gestation and a quantitative change from the ninth through the eleventh day. Exposure of embryos at each of the older ages (ninth, tenth, and eleventh days) resulted in similar patterns of response: the rate of mortality, the amount of growth retardation, and the incidence of malformations all tended to increase as dosage increased. The major difference was in the degree of the response. All the effects were more pronounced after irradiation on the ninth than on the tenth day, and more so on the tenth than the eleventh day. Treatment on the eighth day, however, did not cause malformations at any dosage level, and the rate of mortality did not show a graded increase as dosage increased. This appears to constitute a different pattern of response from that observed when older embryos were treated.

The differences in reaction at the various ages are undoubtedly related to the state of differentiation existing in the embryo at the time of irradiation. It should be recalled that differentiation had not visibly begun on the eighth day; on the ninth day it had progressed to the first stage, namely, formation of the germ layers; on the tenth day active organogenesis had started in a few systems; and by the eleventh day organogenesis was moderately advanced in several systems (fig. 1). The manner in which the susceptibility to irradiation was influenced by the degree of differentiation is unknown. It has been often generalized that susceptibility to irradiation tends to be inversely related to the degree of differentiation. The decline in sensitivity observed as differentiation progressed from the ninth to the eleventh day in the present experiment is in accordance with this generalization. An exception to the rule, however, is the more limited response of the eighth-day as compared with the ninth-, tenth-, and eleventh-day embryos. It appears, therefore, that the diversity of reaction cannot be explained simply in terms of greater or lesser degrees of differentiation.

In view of the particular susceptibility to irradiation of cells undergoing rapid mitosis, the possibility that changes in mitotic rate could account for the changing reactions of the embryos at various ages must be considered. Mitotic counts were not attempted, but it was a matter of direct observation that cell division was frequent and uniformly distributed throughout the undifferentiated eighth-day embryo. A similar frequency and distribution was noted within the three germ layers of the ninth-day embryo. On the tenth and eleventh days, however, cell division became relatively more concentrated in the regions where active organogenesis was in progress. Although these observations are crude, they are, nevertheless, sufficient to indicate that mitotic rate cannot be closely correlated with the results obtained in these experiments. For example, on the eighth and ninth days frequency and distribution of mitosis were similar but the reactions of embryos at these ages were strikingly different. On the other hand, mitoses became increasingly localized in particular tissues between the ninth and eleventh days, yet the reaction of embryos of these ages were basically similar, differing only in degree. Many tissues, such as somites and digestive tract, undergoing rapid proliferation at this time were never affected by malformation. Other structures that were not even recognizable as primordia (true of all organs on ninth day), hence, not specifically proliferating at the time of irradiation, were often later affected by malformation.

Since differentiation occurs primarily at the cellular level, a more adequate interpretation may be reached by considering the various biological reactions to irradiation shown by rapidly proliferating cells. It is generally agreed that proliferating cells, such as comprised these embryos, may show any of three such reactions: (1) death may occur without further cell division, (2) mitosis may be temporarily inhibited but later resumed at an approximately normal rate, or (3) subtle genic alterations (somatic mutations) may occur, but not be manifest until later by faulty differentiation of descendant cells.

The thresholds of sensitivity of these three reactions need not necessarily remain in a set ratio, nor need they change at the same rate as differentiation progresses. The change observed in the embryonic response at different ages can be explained by postulating that, as differentiation began and proceeded, the component cells of the embryos changed their propensity to show one or the other of the cellular reactions enumerated above. For example, the only observed effect of irradiation on the eighth day was smallness of size, except when the dose was raised to 200 r, at which level total mortality occurred. The simplest interpretation is that all cells which were ''hit'' or affected in any manner either died or lost the capacity to proceed with differentiation. The elimination of a moderate number of these totally undifferentiated blastomeres would result in nothing more than a setback in the developmental schedule equivalent to the time required to replace the missing cells. This alone, or perhaps in combination with some mitotic delay in the surviving cells, could account for the retardation later observed. Such destruction of blastomeres presumably could be tolerated up to a critical number, with no consequence other than generalized growth retardation; but destruction beyond this hypothetical point might be expected to cause embryonic death. The absence of an increase in mortality after all smaller doses and the total mortality after 200 r are results compatible with such an explanation. Malformations could not occur if all cells were undifferentiated, since a prerequisite for later maldevelopment would be damage to or loss of cells with predetermined potentialities.

By the ninth day differentiation has begun and no longer are all cells in the embryonic body alike. In addition to having acquired various developmental potentialities, it is probable that different groups of cells have also become variously susceptible to irradiation. Very likely many cells in the ninth-day embryo, as in the eighth-day embryo, were promptly killed by the irradiation, but the destruction of cells already induced to form specific parts of the developing body could be less well tolerated than the loss of totally indifferent cells. This destruction of predetermined cells would lead to tissue deficiencies in later development (one type of malformation) or, if cell death were prevalent in regions of critical potentiality for development, death of the entire embryo would be the consequence. The latter would in part account for the graded mortality rate as dosage increased. It is possible, however, that some affected cells in the ninth-day embryo were not killed, but survived and continued to proliferate more or less normally until some critical event in organ formation was reached. The malformations which later developed in the ninth-day embryo may include some derived from such cells that survived irradiation but bore altered developmental potentialities. As indicated, malformations can result from the deletion of some or all of the cells already induced to form a particular organ, but this mechanism is thought to entail specific deficiency of tissues or parts in the abnormal organ. Several of the observed malformations, such as coloboma, double aortic arch, transposition of cardiac vessels, cryptorchid testes, situs inversus, etc., did not appear to involve such deficiencies. They could be considered to have resulted from aberrant developmental processes initiated by certain of the cells that survived irradiation but suffered genic alterations which only later became evident in defective organ formation. It is also possible that some such ''somatic mutations'' had lethal effects and thus secondarily contributed to the increased mortality among the irradiated embryos.

On the tenth day differentiation had progressed to the point that overt organogenesis was present in certain systems. Despite the relatively great developmental advance, the tenth-day embryo reacted in a similar manner as did the ninth-day embryo, the only significant difference being that a higher dosage was usually required to elicit a comparable response. This may be taken as an indication that the susceptibility of the cells to the various radiation-induced effects was also similar, but that the threshold of reactivity to such effects had risen. By the eleventh day differentiation had reached the stage that organogenesis was moderately advanced in most of the major systems. Nevertheless, the basic response to irradiation was again essentially the same as at the ninth and tenth days, except that the eleventh-day embryo was even less sensitive to a given dose than the tenth-day embryo. Thus the reaction of the rat embryo to irradiation between the ninth and eleventh days of gestation remains qualitatively the same, but during the period sensitivity to the various radiation-induced effects diminishes as differentiation proceeds.

The qualitative change which occurs between the eighth and ninth days of gestation has been shown to coincide with the beginning of cellular differentiation. Although no observations were made on embryos younger than 8 days, it is assumed that they, being equally undifferentiated, would react in a similar fashion as did the eighth-day embryo. As regards the occurrence of malformations, this assumption receives support from the results of other investigators. Russell ('50) observed that the irradiation of mouse embryos prior to day $6\frac{1}{2}$ did not cause maldevelopment. If due allowance is made for the relatively faster developmental schedule of the mouse (approximately 24 hours), the change in susceptibility is reasonably close to that observed in the rat. Likewise, Hicks ('53) was unable to find malformations in young rats irradiated during the first 8 days of gestation, although exposure on the ninth day and subsequently caused a variety of anomalies of the nervous system. Job *et al.* ('35) also observed a change in the susceptibility of rat embryos to maldevelopment induced by radiations, but these authors indicated that the change occurred between the seventh and eighth days. Regardless of the precise time at which it occurs, however, all available evidence indicates that the reactions of both rat and mouse embryos are somewhat different during the first third and during the subsequent parts of the gestation period. The present experiment has defined this difference, not only in terms of the incidence of malformations, but also in terms of the rate of mortality and the degree of growth retardation resulting from irradiation in either period. These differences in reaction have been attributed to changing susceptibilities to the various biological effects of irradiation as the embryo passes from the stage of total undifferentiation to that of beginning differentiation.

SUMMARY

Rat embryos on the eighth, ninth, tenth, or eleventh day of gestation were exposed to doses of 12.5–600 r directly through an abdominal incision in the mother. Lead plates were arranged so that selected embryos could be irradiated while the mother and remaining embryos were shielded. The irradiated and nonirradiated embryos were removed one to several days later, or at term, and compared as to weight, the presence of malformations, and the rate of intrauterine mortality.

Exposure on the eighth day had a qualitatively different effect on subsequent development than did similar treatment on the ninth, tenth, or eleventh day. The only residual effect after doses less than the totally lethal one (200 r) on the eighth day was retardation of growth. Irradiation at the older ages, however, resulted in increases in the number of malformations and the rate of mortality, as well as in retardation of growth, as the dosage increased. This difference in the pattern of response is attributed to a change in the susceptibility of the cells to the various biological effects of irradiation, as the embryo passes from the undifferentiated state on the eighth day into the early stages of differentiation which begin on the ninth day.

Embryos exposed on the ninth, tenth, or eleventh day all displayed a similar pattern of response, but sensitivity to the various radiation-induced effects diminished as the embryo grew older. To produce a comparable response a higher dose was required on the tenth than on the ninth day, and likewise, a higher dose on the eleventh than on the tenth day. These quantitative differences were ascribed to an increasing threshold of sensitivity to the same radiation-induced effects as differentiation proceeds, once it has started.

DISCUSSION

Chairman HAMBURGER: We are confronted immediately with the formidable problem of teratogenesis. I should like to focus your attention on one aspect: To what extent can the radiation effects be attributed to interference with mitotic activity? During this discussion it occurred to me that we should pay considerable attention to other components of embryonic development, such as: morphogenetic movements, inductions and segregations, that is, the orderly apportioning of materials. For instance, it seems to me by no means obvious that such abnormalities as anophthalmia or microphthalmia are due to the loss of cells in the eye anlage. They could be due as well to the abnormal apportioning of medullary plate material, or they could result from an inhibition of the evagination of the optic vesicle, or from abnormal action of the medullary plate inductor, that is, the subjacent mesoderm.

WILSON: First I shall comment on your suggestion that changes in mitotic activity might be involved in producing these results. We did make some crude observations on mitotic rates, enough to indicate that the observed differences in embryonic reaction cannot be attributed to differences in mitotic rate at the time of irradiation. The ninth-day embryo which had begun differentiation showed about the same frequency and about the same uniform distribution of mitosis as did the undifferentiated eighth-day embryo. Yet, you will recall that the large qualitative difference in response to irradiation occurred between the eighth and ninth days of gestation. On the other hand, mitotic activity became more and more localized in regions of active organogenesis on the tenth and eleventh days, but the reaction of the embryo at these ages was only quantitatively different from that on the ninth day. Furthermore, the primordia of the digestive tract showed great mitotic activity at all times, but the digestive tract was not affected by malformations. There was nothing in these appraisals of mitotic activity to indicate that either the rate or distribution of mitosis was responsible for the type of response shown by the embryo.

GENNARO: As excellent as this technique is, it does not eliminate the possibility that there is some effect produced through interruption of the normal physiology of the placenta, perhaps through the decreased oxygenation or impaired nutrition of the embryo.

Have any vitamin A or carotene determinations been carried out on the embryonic tissue itself because of the high incidence of optic deformation?

WILSON: Regarding the apparent similarity between the ocular malformations that were produced by X rays and those produced by vitamin A deficiency, with which I am quite familiar, I can assure you that they are not the same histologically. They are similar only in that on the eleventh day we did get coloboma, and coloboma is a common occurrence after vitamin A deficiency; but even the types of coloboma were different.

The coloboma seen after vitamin A deficiency is characterized not only by the usual persistent choroidal fissure but also by foldings eversion, and cysts of the retina; and of course there is always the associated postlenticular fibroplasia which may be called the hallmark of vitamin A deficiency. I feel that the vague similarity does not justify bringing vitamin A deficiency into the picture. No carotene determinations were made.

Your question about the placenta is good and one that needs to be looked into. Fixed placental material is available but has not yet been subjected to critical study.

Gruenwald: The mechanism by which these malformations are produced is obviously a matter of conjecture. I am not quite sure that abnormal differentiation of cells is involved here, because even if we have fused kidneys, the tissues are still normal kidney tissues. I suspect that we are dealing here with the change in the pattern of growth. I do not think there is too much evidence for abnormal differentiations.

I should like to make another suggestion. Within a day, development in a rat proceeds very much as can be seen by comparing the eighth-, ninth-, and tenth-day embryos. I wonder whether it would be a good idea, if this is feasible, to amputate the top of one of the horns of the uterus and, at the time of the operation, to have the embryos fixed in order to know exactly at what stage you operated.

Wilson: As Dr. Gruenwald suggests, the fused kidneys which we observed seemed to contain normal renal tissue as far as one could tell by examination under low and moderate powers of magnification. We did not do cytological studies. I did not intend to imply that there had been abnormal differentiation within the kidney primordia. My suggestion was that perhaps such malformations as these — malformations which are not in any way preformed or simulated in prior development — are perhaps the result of somatic mutations. The malformations could have resulted from deviations in the developmental pattern of the whole region, due to some basic alteration in the chromosomal or genic makeup of precursor cells. The developmental deviations could consist of abnormal patterns of growth, as you have suggested, but possibly also of abnormal differentiation somewhere in the caudal end of the body, as I have suggested.

Yes, I think it would be possible to remove an embryo at the time of operation to determine the exact stage of development.

Brunst: What is your explanation of some of the numbers in your slides dealing with mortality after irradiation, such as 8 days, 0%; 9 days, 11%; 10 days, 14%; 11 days, 0% — all after a dosage of 50 r? It is not clear to me.

Another question: Are the sizes of cells in the irradiated tissue normal? I ask because many observations show that the size of cells in irradiated tissue is not normal. Are these malformations the result of damage to the differentiation process or the result of inhibitions of growth and cell damage?

I was glad to hear about these types of defects in the development of eyes; this is in accordance with my data. In the matter of development of the retina after irradiation, did you observe some malformation in the retina and, particularly, in the rods and cones layer? Of course there are some papers which described the special sensitivity of the retina. Is this true according to your material?

I think it is very interesting to investigate the effect of local irradiation with your method. Thin lead strips can be used to protect part of the embryo, and in such a way the effect of X irradiation may be investigated.

Wilson: I think you have taken the data out of context, hence the apparent inconsistency. Mortality rates in rat embryos are highly variable, as you noted from the control data. Rather than concentrate on a single dosage or age group one has to compare over-all effects when a graduated series of doses or several age groups are used. That is what I tried to do.

Regarding your second question, I have no data on the size of cells in irradiated as compared with nonirradiated animals. Likewise, I am unable to answer your question on the detailed structure of the retina.

I shall not attempt to repeat all my speculations on the manner in which the malformations were brought about but shall simply say that I feel that both abnormal differentiation as well as abnormal growth patterns may have been involved.

WEISS: As to the question of mitosis, of course we know, more or less, that the process of cell division falls into two phases — the synthesis of protoplasm (increase of the cell) and the triggering off of the act of division. Is there perhaps a difference in the periods before and after 8 days so that in the earlier period there would be something corresponding more nearly to the act of cleavage, where previously synthesized material is broken up into cells; whereas, only during the later period the process of increased synthesis would be involved? Might this not explain this rather sharp cut between the two processes?

Then I have a purely terminological question in regard to what Dr. Gruenwald said. A fused kidney is described as a teratoma. That only means we are perhaps looking at the more conspicuous, but not at the crucial, part of the system, because the fused kidney itself may have nothing wrong with it. The effect may be on the surrounding tissues. I think it is entirely illegitimate to pick out arbitrarily, so to speak, one part of the system, in this case the kidney, and focus on it just because it stains better or because it is more conspicuous, and imply that it is the primary site of action. The primary defect may be in the surroundings. In that case kidney fusion is merely a secondary consequence.

A third point, there was this remarkable falling off of sensitivity to teratogenic effects with age. Is that perhaps merely a technical problem correlated with the increased mortality? Since there is increased mortality with age, by that time those embryos which did survive were less likely to show teratogenic effects because if they had been defective they would have died and been listed among the dead ones. Therefore the question arises whether this is merely an illusion of the figures.

Wilson: You have suggested the interesting possibility that the change in type of embryonic reaction between the eighth and ninth days may be related to a change in the synthesizing activities of the cells. I can think of nothing in my data that would speak against it. I do not agree with your further suggestion that the falling off of the embryo's sensitivity to the teratogenic effect of irradiation was due to an increase in mortality as the embryos grew older. Mortality did not increase in older embryos. Actually, sensitivity to the lethal effect of irradiation diminished with age almost as rapidly as did sensitivity to the teratogenic effect.

Butler: I am interested especially in the remarks about the relation effects of irradiation on cell proliferation and on differentiation, since well before the advent of the atomic age, I was interested in the effect of radiation on various aspects of growth. It would be especially interesting if, in this type of work on mammals, individual organs or individual areas were chosen for considerable detailed study of the effect of radiation on differentiation. In this type of study we have found that irradiation of the limb of an amphibian embryo, just at the time the limb area is being determined and differentiation is about to become evident, will not greatly interfere with the development of a limb. But the limb which develops will not be a normal limb; differentiation of certain limb structures will be interfered with. A limb of considerable size may develop, but be completely devoid of digits. We are dealing, in other words, with the influence of radiation on morphogenetic activity.

Similar experiments can be done on limb regeneration. When a urodele limb is amputated a blastema forms and regeneration ordinarily progresses rapidly. By irradiating a regenerating limb one can govern, so to speak, the type of morphogenetic expression. For example, by irradiating at the proper time with proper dosage, a two-digit hand, instead of a three-digit or four-digit hand can be produced. It is possible in such experiments to permit mitotic activity and increase in cell number, but at the same time rather precisely to govern morphogenesis and differentiation. It would be very interesting and instructive if experiments of such a nature could be carried through on organs of the mammalian embryo — such organs as the eye, for example.

In Dr. Wilson's results we noted that in the mammal irradiation was done at such a time as to prevent the development of the forelimb, and yet in the same embryo a fairly normal hind limb developed. The forelimb in the normal sequence of events is established earlier than the hind limb.

KIMBALL: I think there are great difficulties, from the genetic point of view, in the somatic mutation hypothesis. Viable somatic mutations should be induced at all stages of development, not only after the eighth day. Furthermore, the mutations should be scattered at random among the cells. Cell-lethal chromosome aberrations might be considered as a cause, but not somatic mutation in the usual sense.

WILSON: Many of the questions seem to hinge around my conjecture regarding somatic mutations as possibly being involved in producing some of the malformations. That is my naïve way of looking at it. I find it difficult to see how all of the effects can be attributed simply to cell death or to slowed mitotic rates. Since I am neither a geneticist nor an experimental embryologist, I am really not competent to discuss the matter critically.

HESTON: I am, of course, interested in the comparison of these abnormalities that occur following treatment, with those that occur without treatment and have a genetic basis — in particular, the absence of the kidney. In the highly inbred strain A × C 9935 (Irish) approximately 35% of the animals lack one kidney, or in some cases, have a poorly developed kidney. It would appear that this is another character the incidence of which is established in the inbred strain by the genetic constitution of the strain.

But in this particular condition, not only is the kidney missing but also various portions of the genital tract. In the females, the corresponding horn of the uterus or a proximal portion of the horn, or the ovarian capsule may be missing, whereas in the male, the corresponding epididymis or vas deferens may be missing. In your treated animals was the absence of the kidney accompanied by such abnormalities of the genital tract?

WILSON: Yes, associated with the renal anomalies there occasionally were defects in other urogenital organs. Males with agenesis of one kidney usually had cryptorchid testes and faulty or absent male ducts on the side of the missing kidney. Aside from this, however, I do not recall other situations in which renal anomaly was regularly accompanied by defects in the genital system.

CARTER: The suggestion that somatic mutation may play any but a very minor role in the induction of embryonic abnormalities seems open to question, at least until more is known about the developmental mechanisms involved. Horseshoe kidney may be taken as a case in point. The mechanism by which it developed in Dr. Wilson's rats is unknown; but the development of a similar condition has been investigated in a genetically abnormal mouse strain. There it was found to be the remote result of an abnormality of the umbilical arteries, which led to fusion of the kidneys through mechanical interference with renal migration. It must at least be borne in mind, therefore, that the organ in which an abnormality is seen is not necessarily that in which the first change occurred.

WILSON: I agree that the structure showing the greatest deviation from normal is not necessarily the one first affected. You are right in saying that the mechanism by which the horseshoe kidneys developed is unknown, but in studying these I paid particular attention to the matter of the umbilical arteries. There was no indication of abnormal size, location or proximity of these vessels after the kidneys had fused, but irradiated embryos have not been studied at ages when the kidneys were in process of passing between the umbilical arteries.

L. RUSSELL: I should like to take issue against interpreting these abnormalities by the production of somatic mutations (and thus add my criticism to Dr. Kimball's). First of all, it is necessary to assume directed mutations in order to give similar abnormalities in different irradiated animals. Such an assumption is against the known facts of radiation-induced mutation.

Secondly, it is not possible to account by gene mutation for the frequency with which abnormalities follow irradiation of embryos. Even taking mammalian germinal mutation rates, which apparently are higher than *Drosophila* rates (Russell, '51), and assuming the same rates in somatic cells, 200 r would give about two mutations per body cell. With the average degree of dominance being presumably quite low, only a very minute fraction of the cells would thus be affected in the diploid somatic tissues.

I believe that it is possible to postulate death of cells as the primary cellular effect of radiation in embryos. Such a primary change would touch off exceedingly complex chains of development and it is not necessary to assume that the finally observed abnormality is in the nature of a deficiency of tissue. These points are discussed in more detail in my paper (This Supplement).

I should also like to comment on the general question of mitotic rate studies. I believe it would be most important to compare rates of different regions of an embryo at any given stage and correlate the findings with abnormalities observed to follow irradiation of that stage. On the other hand, it seems to me that only little could be gained from looking for possible over-all differences between an eighth- and a ninth-day embryo, as Dr. Wilson has suggested. The response of the former seems certainly to be due to the great regulatory powers of the early mammalian embryo and corresponds closely to the mouse results for days ½ to 5½ postconception (Russell, '50).

Finally, I should like to answer Dr. Brunst's question about the feasibility of irradiating only selected parts of embryos. Raynaud and Frilley, in France, have directed a very thin pencil X ray to the head region, particularly to the developing pituitary of rat embryos.

EDDS: Since the issue of possible differences in mitotic rate has been raised, it is important to draw attention to the work of Corliss ('53). His observations of the mitotic activity in the rat embryo during the ninth day of development bear on the results Dr. Wilson has reported. Corliss finds that during the ninth day, there is a progressive increase in the number of dividing cells per unit volume of tissue. The increase is small during the early part of the day but quite pronounced during the second half of the day. Mitotic activity is not differentially localized in any one region, however, but is uniformly distributed through the embryo.

WAELSCH: It is very interesting that Dr. Wilson's irradiations of rats on the eighth day of pregnancy produce either complete lethality, i.e., death of the embryo, or newborns which are perfectly normal. Dr. Wilson, have you looked at those embryos just before or after death; and did they perhaps appear abnormal in any way just before they died? It is really not too surprising that those embryos which do survive appear perfectly normal; the reason for it is that the rat embryo at that stage is probably still in a condition in which its regulatory power is strong enough to overcome the damaging effect of the X rays in all its developmental systems, in contrast to later stages when regulatory power has become considerably weaker.

Have you looked at any of the stages of the developing nervous system preceding the appearance of exencephaly and did I understand you to say that the brain was fused with the overlying ectoderm? I wonder whether the neural folds do close before this condition, i.e., exencephaly arises, or whether they never close, or whether there has not been a chance to look at embryos before the abnormality appears?

WILSON: As to your question about dead embryos, whenever the embryo was still in a sufficiently good state of preservation to make any sort of histologic study possible, we did section and study it. In such eighth-day embryos we have found nothing that we could identify as a malformation. Your suggestion that the reparative power of the eighth-day embryo might be superior to that of the older embryos is certainly a possibility.

The neural folds were seen to be closed in all early embryos that were examined. Frequently on the second, third, and fourth days postirradiation, however, a closed neural tube was seen to be fused with the overlying ectoderm. Thus we feel sure that the defects in the brain and the spinal cord were not related to primary failure of closure of the neural tube, but were somehow the result of fusion between ectoderm and neural tube.

HICKS: In answer to those last two questions, we have given 400 r to eighth-day embryos *in utero* and usually found them to be necrotic some hours later. I shall show in my paper (This Supplement) that in later embryos selective necrosis occurs following irradiation; e.g., on the ninth day the anterior neural folds are vulnerable.

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THE EFFECT OF IONIZING RADIATIONS ON AMPHIBIAN DEVELOPMENT

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FOUR PLATES

INTRODUCTION

Rigidly delimited, discussions relating to the embryo should begin with the fertilization process and end with the achievement of the independently viable progeny which resembles the parent. In amphibia, the larva or tadpole must still undergo metamorphic changes to become a frog, toad, or salamander and, in the experimental field of regeneration, it seems to exhibit some of the characteristics normally associated with embryonic development. The radiosensitivity levels are so different for the embryo, the tadpole, and the frog that, radiobiologically, they must be considered independently. Stearner ('50) stated that the LD_{50} for the adult frog is 700 r and Allen, Schjeide, and Hochwald ('51) found 10,000 r to be the LD_{50} for *Rana catesbiana* tadpoles. In this discussion it is proposed to include also some references to the effects of ionizing radiations on the gametes insofar as such radiations alter subsequent embryonic development. Background information from the summary chapter by Butler ('36) in Duggar's book will be taken for granted.

RECENT LITERATURE

The gametes. Interest in the "Hertwig Effect" has been revived recently by Briggs *et al.* ('51). The "Hertwig Effect" is simply the elimination by ionizing radiations of the syngamic or genetic function of the gametic nucleus, resulting in haploid development. Such development as occurs is essentially parthenogenetic, and may be either androgenetic or gynogenetic. The egg-activating properties of the spermatozoon were not altered even by an exposure to 120,000 r of X rays, so that 100% fertilization was achieved by such irradiated sperm. This was clearly demonstrated (Rugh and Exner, '40) by using irradiated bullfrog sperm to activate leopard frog eggs, these eggs developing further than those fertilized by nonirradiated sperm of the foreign species. Parthenogenetic (haploid) development was achieved in 93% of the eggs exposed by the simple process of eliminating, by X irradiation, the nuclear contributions of the sperm.

Briggs *et al.* ('51) studied such eggs cytologically and found that 86% were definitely haploid and less than 1% were diploid. Cleavage was normal but delayed. In those studies the unirradiated female (egg) nucleus was removed from the inseminated eggs before syngamy could be accomplished, leaving the X-irradiated sperm alone in the normal egg yolk and cytoplasm. Such eggs showed delayed cleavage and many of the blastomeres were entirely without chromosomal material. This suggests that the mechanism for cleavage may be achromatic. The chromatin-bearing and chromatin-free blastomeres appeared to be bunched separately in the partial blastulae at 24 hours. When transplanted to a normal host environment the achromosomal cells failed to survive any better.

Duryee ('47), with his very refined micromanipulation techniques, examined and transplanted irradiated chromosomes and nuclei, with the following findings:

1. Ovarian eggs from salamanders or frogs, when X-irradiated with 500–60,000 r in graded steps, showed progressive stages of pycnosis. After 3000 r some 98% of the small eggs showed intranuclear damage within 10 days.
2. Criteria for irradiation injury were: fragmentation of the chromosomes, loss of lateral chromomere loops, colloidal changes in the nucleoli, sol-gel changes in the intranuclear spindle substance, and localized cytoplasmic flocculation.

3. Nuclear components *in situ* were not equally radiosensitive. Nucleoli were vacuolated at 1000 r; chromomere loops were dissolved after 2000 r; chromonemata were broken at 3000 r; and spindle substance was solvated at 50,000 r. These components, when individually isolated from their cytoplasmic environment and then X-irradiated, proved to be markedly radioresistant. This suggests an indirect effect of ionizing radiations, by way of the cytoplasm and/or the yolk.

4. Exposures to low temperatures (5°C.) delayed nuclear injury from X irradiation up to 18 days but did not prevent eventual pycnosis when the animals were brought to 23°C. Higher temperatures accelerated the appearance of the radiation injury.

5. Isolated egg nuclei were highly resistant to X-ray damage as compared with nuclei *in situ.*

6. Microinjections into nonirradiated eggs of cytoplasmic material from irradiated eggs induced typical nuclear radiation damage. Chromosomal and nuclear injuries were produced by exposure of isolated normal nuclei to X-irradiated cytoplasmic brei.

7. Cellular radiation damage was caused by (1) radiochemical changes in the cytoplasm, (2) accumulation of metabolic toxins from irradiated nuclei, and (3) rapid transmission of toxic substances into the nucleus at usual laboratory temperatures.

Adult salamanders *(Triturus pyrrhogaster)* were exposed to X rays of 500–50,000 r (Rugh, '50b) and studied for the immediate and delayed effects on the meiotic chromosomes in spermatogenesis. Formed chromosomes immediately became less viscous, more fluid and sticky. They tended to flow out along the spindle fibers, lose their identity, and become clumped. The spindle fibers became so coated with fluid chromatin substance that they too became chromatic and reacted to the Feulgen stain. These changes were immediate. At lower X-ray doses the end point of radiation change was the pycnotic nucleus, seen most frequently in the secondary spermatocytes. Chromosomes exposed to

5000 r never returned to their normal structure. The interchromosome stages showed fragmentation of the linin network. After 48 hours the network lost its identity and the nucleus contained merely a homogeneous mass of semichromatic material. It was concluded that all stages of spermatogenesis are radiosensitive. The effects may not be apparent immediately. If mitosis is not inhibited, all cells will be so affected during that process.

The embryo. Rollason ('49) X-irradiated eggs of *Rana pipiens* in various stages of maturation and fertilization and found that the newly fertilized eggs were the most radiosensitive, followed by the uterine, and then the ovarian eggs. The newly fertilized egg is digametic with motile pronuclei in syngamic convergence. The uterine egg is suspended in the second maturation metaphase while the ovarian egg is in a premature stage with elongate, rather diffuse chromatin material. Rollason found that spermatozoa were more radiosensitive than the eggs, as measured by the effects on embryonic development. Although cleavage percentage was affected, the cleavage rate and pattern were not altered by X irradiation of the egg. A delay of 24 hours between irradiation and fertilization of the uterine egg excluded evidence of any recovery changes. The types of abnormalities that followed exposure of the egg were similar to those which followed the irradiation of sperm.

A single exposure to 10,000 r of frog embryos between stages 18 and 25 killed all within 6 days, and 50,000 r killed all within 3 days at laboratory temperatures of 23°–25°C. (Rugh, '49, '50a). The later stages in this series were more radiosensitive than the early ones, due probably to the development of the excretory and respiratory systems. Growth was impeded by 10,000 r and abruptly stopped by 50,000 r. Exposure at different stages of early development elicited different developmental abnormalities such as lordosis following exposure at stage 18, belly edema at stage 21, and head blisters at stage 25. At stage 25 there was no belly edema or lordosis, normally found after irradiation of the earlier stages. These regional sensitivities are explained on the basis of heightened regional activity (differentiation) at the time of exposure. The lordosis was caused by exposure at a time when the central nervous system and axial (notochordal) skeleton were being laid down. The belly edema occurred in tadpoles irradiated at development of the coelomic cavity and the head blisters followed exposure during formation of the anterior lymph spaces and the internal gill chamber.

Radiosensitive organs of an amphibian embryo, when transplanted to the healthy environment of a nonirradiated host, may outlive their life expectancy of the original environment (Perri, '50). A transplanted eye, for instance, destined to succumb in 3 days in the intact embryo may live as long as the normal, healthy, nonirradiated host into which it is transplanted. Embryonic radiation death, therefore, even as adult death, may be due to radiation damage to only one especially radiosensitive but vital organ system. This regional sensitivity, particularly in the embryo, may be associated with the fact that at the moment of exposure certain regions are dynamically changing into functionally distinct tissues or organs by the process of differentiation. More recently, Piatt and Raventos ('53) exposed the developing ear, medulla, and forelimbs of stages 15 and 16 of *Amblystoma* embryos to 200–600 r of X radiation, and subsequently transplanted these organs orthotopically to nonirradiated embryos at stage 27. They found the degree of differentiation to be inversely proportional to the amount of radiation. No forelimbs developed from the 400-r or 600-r grafts. Following exposure to 200 r the grafts grew, but were retarded and reduced in size. These authors stated that irradiated tissue grafted to normal embryos developed abnormally but attained a higher degree of differentiation than under the conditions of total-body irradiation. Their embryos exposed to 200 r died by the twenty-ninth day, those exposed to 400 r died by the fourteenth day, and 600 r killed all by the twelfth day.

The tadpole. Amblystoma larvae measuring 22 mm were exposed to 15,000 r (Rugh, '49) and most survived for 10 days. Some were killed at 7 days and it was found that all nuclei which were in the process of mitosis at the time of irradiation became pycnotic and others were inhibited from initiating mitosis. In other words, at this high level of exposure, even with elapsed time, no mitotic phases appeared, and pycnosis seemed to involve only those cells with distinct chromosomes. Since the relative number of pycnotic cells was not excessive, it was presumed that all premitotic phases had been abruptly prevented from entering mitosis. Cartilage and muscle cells at this stage were the best developed of the various differentiated tissues, and these were the least damaged by the X radiation. The neuroblasts of the developing central nervous system and the related sense organs, such as the eyes, were the most radiosensitive. Neuroblasts were sloughed off into the brain cavity and neurocoel, and were largely pycnotic. This seems to be because they were actively differentiating at the time of exposure. Thus the tissue sensitivity of the embryo is quite different from that of the adult, where the nervous system is presumed to be relatively radioresistant.

Tail development in axolotl larvae from 5 to 12 days of age was inhibited by local exposure to X rays (Brunst, '50b). After 35–40 days, Brunst differentiated between zones of inflammation (acute primary reaction), zones of stimulation (overcrowded cells in mitosis), and zones of inhibition (absence of mitosis). He found giant polyblast macrophages which were phagocytizing the cells that had been damaged by irradiation. By 85 days all signs of the so-called acute primary reaction were gone and the surviving cells had recovered their normal appearance but had lost their capacity for cell division. Secondary tails occasionally grew from the proximal portion of the original tail, but were always smaller than the normal tail. In another experiment Brunst ('50a) locally X-rayed the limbs and lumbosacral region of axolotl larvae (17–23 mm), and found that the treatment inhibited limb development and prevented any formation of the pelvic girdle. Some development of the limb occurred if radiation was imposed after the initial appearance of its rudiment. Brunst says: "It is difficult to postulate differentiation that might occur apart from mitosis, although this possibility cannot be denied. It does not appear to be common for cells to undergo metamorphic changes independently of mitosis."

Brunst *et al.* ('52) spot X-rayed (2000–4000 r) the lower jaw of 20- to 60-day-old salamander larvae, stopping mitosis and causing the enamel epithelium to disappear. New teeth never developed, showing that the mechanism for tooth formation and tooth maintenance were destroyed. Allen and coworkers (Allen, Schjeide, and Hochwald, '50, '51; Allen, Schjeide, and Piccirillo, '51b; Allen *et al.,* '52) have recently studied the hematopoietic cells of the anterior kidney of the frog tadpole with respect to irradiation effects. Tadpoles kept at 5.5°C. or lower during irradiation were not protected insofar as the hematopoietic elements were concerned. This was demonstrated when exposed animals were returned from 5.5°C. to normal laboratory temperatures. Allen, Schjeide, and Hochwald ('50) were the first to show that the temperature of tadpoles at the time of X-ray treatment made little if any difference in the destructive processes that ensued, but that the destructive effects after irradiation could be delayed by low temperatures.

In 1952, Gojmerac and O'Brien exposed larvae of *R. catesbiana* and *R. pipiens* to 10,000–15,000 r of X rays at 23° and 3°C. and, subsequently maintained groups of them at both of these temperatures. They found that the temperature during X irradiation was without apparent effect but that the postirradiation temperature determined the time of onset of the damage. This inevitably occurred, however.

Allen, Schjeide, and Hochwald ('51) found *R. catesbiana* tadpoles to have an $LD_{50/30}$ days of 10,000 r. Nevertheless, 500 r caused very extensive destruction of the hematopoietic tissues; this could be delayed but not nullified by maintaining the tadpoles at a low temperature. They pointed out that the hematopoietic tissue of tadpoles exposed to 500 r might not exhibit the effects for some time, awaiting such dynamic changes in the cells as mitosis. In a later study (Schjeide *et al.*, '52), the metabolic activity and rate of mitosis were emphasized as the factors controlling the expression of radiosensitivity. They stated, "We find that the tadpole hematopoietic tissue appears to be susceptible to 500 r in direct correlation with the amount of cell division allowed to proceed, following the irradiation period. Cells in prophase at the time 500 r of X irradiation is applied are destroyed directly." Cells held in late prophase by colchicine were destroyed by 500 r of X rays. They further stated, "Our evidence seems to indicate that the primary change is inflicted in a very high percentage of the hematopoietic cells at the time of irradiation, but this damage is not visible as destruction until these cells begin to divide." Here again the evidence of radiosensitivity seems to be manifested only during the dynamic changes of mitosis.

The major thesis in these studies seems to be simply that the rate of mitosis is controlled by the temperature and that X radiation-damaged cells are destroyed as they proceed to prophase; hence the evidence of destruction is increased with an elevation of temperature and the consequent acceleration of mitosis. This simply means that cells may be damaged by X rays but do not give morphological evidence of that damage until mitosis ensues. At high X-ray exposures the amount of hematopoietic cell destruction exceeds the number of cells in mitosis. Allen *et al.* ('52) were able to detect destruction among the hematopoietic elements after doses as low as 25 r, observing graded effects with increasing doses used up to 10,000 r. After 250 and 750 r, for instance, mitotic figures were rarely seen before the fifth day postirradiation. After 1000 r all hematopoietic cells ceased to divide by the thirtieth day. Following 500 or 10,000 r those tadpoles which had been raised at 4.5°, instead of 20°C., showed a marked reduction in the pycnotic hematopoietic cells, but at 10 days the number of such cells following the two extremes of radiation exposure were about equal — and ten times as many as seen at the end of 1 day. Schjeide *et al.* ('53) found an inverse ratio of exposure to white blood cell count, the lymphocytes displaying the fastest disappearance and the thrombocytes the slowest. They used this information to estimate the life span of the various blood cell constituents. Their estimates were: lymphocytes, a few hours to a few days; neutrophils, 10 days; basophils and eosinophils, 16 days; and thrombocytes, 50 days.

Allen, Schjeide, and Piccirillo ('51) found that anoxia, applied either during or after irradiation, markedly reduced the immediate radiation destruction in tadpole cells. Probably the low temperature, anoxia, and colchicine effects were each achieved by delaying the process of mitosis. They all delayed but did not prevent X-ray destruction. Hematopoiesis practically ceases in the tadpole kept at 5°C. or in tadpoles exposed to more than 100 r of X rays (from Schjeide, personal communication).

Regeneration. Since 1933 Brunst and his coworkers have published some twenty-one titles on amphibian regeneration as it was affected by X irradiation. Many of these studies dealt with the limbs of immature and adult aquatic axolotls but in some of the more recent studies the early larval stages have been used. Brunst stated that sublethal exposures may prevent growth, regeneration, and development and that tissues or organs which include X-ray-inhibited mitoses, may still persist for a long time in a secondary stable state. This theoretical concept, which implies ultimate recovery from radiation effects, is certainly new. Brunst believes that differentiation can proceed in spite of the radiation suppression of cell division. This concept is difficult to accept, especially since cells in the process of differentiation have proved to be more radiosensitive than simply dividing cells. Ontogenetically, differentiation is accomplished simultaneously with cell division, and it is difficult to see how a cell once differentiated can become otherwise differentiated without an intervening process of dedifferentiation and redifferentiation accompanied by mitotic activity. Such plasticity of already differentiated cells is demonstrated. Brunst seems to believe that the regenerating process is entirely local, while some others believe the organism as a whole exerts some influence on the regenerating process. Attention is directed to the summary paper by Brunst ('50).

A few generalizations may be in order. Ionizing radiations have been used in studies on regeneration because of their demonstrated effects on embryonic undifferentiated tissue, and because regenerating blastemas have been considered to be basically similar to proliferating embryonic tissues. The isolated but unverified suggestions that radiations might accelerate the growth process have encouraged their use at low levels in regeneration studies. Brunst ('52) has stated: "A number of investigations have been described in which many cases of roentgen stimulation have occurred. For instance, the stimulation of the development of additional tails or supernumerary limbs was observed in axolotls following roentgen irradiation. It is possible that, in many cases, this stimulation is a result of the effects of disintegration products of destroyed irradiated cells. This possibility that the roentgen stimulation was induced by necrohormones or wound hormones was considered." Until it is thoroughly substantiated, this is indeed a rather dangerous concept to advance in light of the ever-widening use of various levels of radiation, local and diffuse, to human beings of all ages. Clinically, there is no evidence of any stimulating effect of ionizing radiations. It can hardly be said that edema, erythema, or hemorrhage is evidence of radiation stimulation. It must be pointed out that supernumerary limbs can be produced easily by dividing the blastema in any of a number of ways. The potency of a limb anlagen, or blastema, is unitary only until it is divided, and the dividing agent might well be a beam of ionizing radiation. Certainly the cut surface of an embryo or a young adult organism provides cells which are physiologically different from the adjacent cells and which may conceivably have different radiosensitivities. The exposure to ionizing radiations of a mutilated or cut surface which is actively proliferating might be expected to produce a reaction in much the same manner as would exposure of the rapidly cleaving stages of the early embryo. Such a surface should be hypersensitive and multipotent. Since it is still doubtful that there is any stimulation of mitosis by ionizing radiations and since there is abundant proof of the deleterious and retarding effects of ionizing radiations on cleavage and mitosis, it might be predicted that ionizing radiations would impede or even inhibit the normal processes of regeneration. At subinhibitory levels radiations could well induce teratologies in regenerating tissues. This should be particularly true when the radiations are locally applied. In the case of a low exposure of an entire organism, special mitotic impedence by ionizing radiations of regenerating stumps would also be predicted on the assumption of hypersensitivity of the proliferating cells at the cut surface. There is no doubt that regenerating organs can be made to develop abnormally, following exposure to ionizing radiations, but these same abnormalities can be produced by other means, even by inert implants, so that the reacting system is the more important consideration. ''Stimulation of mitotic activity by roentgen irradiation'' (Brunst, '52) cannot yet be accepted as proven.

CURRENT RESEARCH

The first data to be presented relate to the dose necessary to kill tadpoles under acute X irradiation. Groups of ten tadpoles of 1 month of age and also some of premetamorphic stage were exposed to parallel sources at 2200 r/minute (110 kvp and 30 ma, no filtration) and were observed at 5000-r intervals after 100,000 r had been accumulated. In $1\frac{1}{4}$ hours all the older tadpoles were dead at 165,000 r and all the younger tadpoles died from 187,000 r in 1 hour and 25 minutes. In each group there was less than 20,000 r difference in radiation dose between the first and last individuals to die. The slightly greater tolerance of the younger tadpoles may have no significance. Nevertheless, the dose necessary to kill older tadpoles is higher and the time shorter than were found necessary to kill mature mammals such as mice and hamsters.

The causes of acute radiation death are not thoroughly understood. Suspecting that histochemical changes might be seen prior to the histopathological, we irradiated other tadpoles acutely and preserved them immediately in cold Rossman's fixative. The sectioned material was examined for the presence of mucopolysaccharides by the method of Hotchkiss ('48).* Control tadpoles of the same age and source were similarly fixed and treated for periodic acid-Schiff (PAS) reaction. Animal polysaccharides such as glycogen, are oxidized by periodic acid to give polyaldehydes which yield colored compounds with the Schiff's reagent, fuchsin sulfite. Tissues roughly treated in this manner lose a great deal of their cytological detail but the over-all abundance of mucopolysaccharides can be determined relatively.

Examination of the tissue of the controls and the tadpoles killed under acute X-ray exposure indicated an abundance of mucopolysaccharides toward the periphery of the central nervous structures — the brain and spinal cord (plate 1). In these regions the mucopolysaccharides were greatly depreciated in the acutely irradiated tadpoles. There also was considerable loss from muscle, from both the inner and outer zones of the retina, some slight loss from kidney tubules and gut epithelium, but very little from the liver. The most drastic changes appeared to be in the central nervous system, which finding should be correlated with histological changes to be described later. Obviously, it is difficult to quantitate these effects, but a direct comparison of similarly made photographs of comparable organs of control and X-ray-killed tadpoles clearly indicates an immediate depletion in the mucopolysaccharides. Similar studies on the liver glycogen of adult mammals and urodeles have indicated a rapid depreciation following X irradiation (Levy and Rugh, '53). We are currently making similar tests on the tadpole for protein changes, based on the bromphenol blue method of detection.

Recent studies have shown that certain –SH-containing amino acids give mammals protection against ionizing radiations when they are injected just before exposure (Patt and

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* With the help of Dr. Barnet Levy.

Swift, '48; Bacq *et al.*, '51). Obviously, it occurred to us that these aquatic forms might quickly absorb soluble amino acids and be afforded some radiation protection thereby. Since 1-month-old tadpoles were known to tolerate X rays in the vicinity of 10,000 r for at least 2 weeks, two doses of 11,000 and 16,500 r were chosen for a test of the possible protective effect of cysteine and cysteinamine against ionizing radiations. It was first necessary to determine the concentration of the dissolved amino acids which the tadpoles could tolerate for a period of 1 or more hours. This proved to be 0.01% cysteine at pH 7, and 0.05% cysteinamine, all made up in Great Bear Spring Water.

The tadpoles, all from the same source, were segregated into groups of fifty each. Two sets of controls were maintained, one exposed to X rays alone and the other to the amino acids alone, each set for period of 1 hour. The experimentals were exposed to the amino acids for 5 minutes, X-irradiated (5 or 7.5 minutes), and then placed in fresh spring water. All were treated similarly with respect to temperature, food, and water changes.

The results were inconclusive but suggestive. None of the amino acid-treated controls died. All of the X-irradiated controls died within 10 days. Only the cysteinamine-treated and X-irradiated tadpoles (at both 11,000 and 16,500 r survived longer than the X-irradiated controls, and these for only 13 days. The immersion of tadpoles in the solutions before and during irradiation did not work adversely for them, and only the cysteinamine appeared to give some slight protection by extending the life span following X irradiation. This investigation should be extended, since such biological test material is abundantly available. The doses used should be more nearly at the LD_{50} level for the stage studied.

For radioembryological studies, amphibian material is particularly useful. It is available throughout the year in abundance by pituitary-induced ovulation and, since each frog can produce upward of 2000 eggs, this number of essentially similar genetic embryos can be made available at any time.

It may be of interest to point out that about 10 years ago we found that the gonad-stimulating potency of the isolated adult frog anterior pituitary gland could not be altered even with an exposure to 100,000 r of X rays. Such glands, quickly frozen, retained their potency for over a year. Allen *et al.* ('53) have found that 20,000 r to the recently metamorphosed toad did not alter the thyroid-stimulating potency of its anterior pituitary as determined by subcutaneous transplantation to tadpoles of the same species. These tadpoles were accelerated toward metamorphosis well ahead of the controls. The frog pituitary gland, whether in an adult or recently metamorphosed stage, is as resistant to radiation damage as acromegaly or sterility studies on the adult human pituitary seem to show.

The following data on the amphibian embryo are essentially qualitative, although quantitative inferences can be made. Therefore, observations on the various stages from the uterine egg to the 2-month-old tadpole will be made chronologically.

The uterine eggs of the frog are all in the same stage of maturation, namely, metaphase of the second maturation division. Such eggs are very much more radiosensitive than the spermatozoa of the same species, as determined by the effect on early development. According to Rollason ('49), only 50% of such eggs exposed to 200 r of X rays developed normally. Exposures as high as 42,000 r did not prevent successful fertilization of such eggs by normal spermatozoa of the same species and in our experiments uterine eggs exposed to 1500 r all attempted gastrulation. Twelve per cent reached the yolk plug stage, while the remaining 88% became exogastrulae with everted yolk. All these were dead by 72 hours, however, but the lower exposure of 300 r allowed some eggs to develop quite normally. Only 20% of the uterine eggs exposed to 300 r became exogastrulae; the remaining 80% developed normally and as rapidly as the controls for 96 hours, and then many became abnormal and died. Some of the survivors were merely amorphous masses, some (20%) resembled parthenogenetically produced larvae, and a few

(2%) developed quite normally into tadpoles. It is obvious, therefore, that irradiation of the uterine egg will not affect its fertilizability, or its early cleavage, but doses of 300 r or more will produce nonviable teratologies (pl. 2).

Fertilization of the frog's egg can be controlled and timed to research convenience. Eggs inseminated at laboratory temperatures of 23–25°C. will give off their second polar bodies within 25 minutes after insemination. Syngamy follows, and the first cleavage occurs in 2½ hours. As the polar body is being extruded, the male and female pronuclei slowly migrate toward each other through the cytoplasm. Rollason ('49) reported that only 50% of the newly fertilized eggs developed normally following exposure of 150 r of X rays.

Eggs were X-rayed within the period of polar body extrusion (19 minutes) at doses ranging from 300 to 1500 r. The time and manner of the first cleavage were not altered by any exposure used. However, by 24 hours there was evidence of delay in development to a degree somewhat correlated with the exposure, beginning with the lowest dose of 300 r. Some of the fertilized eggs exposed to 300 r reached the yolk plug stage simultaneously with all of the controls; those exposed to 900 r were retarded to the dorsal lip stage and those exposed to 1500 r were only in the late cleavage stage (pl. 3). By 48 hours it was evident that these were terminal stages of development at the respective dose levels. Two per cent of the 600-r eggs tried to gastrulate; 16% of the 300-r eggs did achieve gastrulation, but very few developed into any semblance of larvae. Development beyond gastrulation was not so successful as in irradiated uterine eggs, most of the survivors dying as exogastrulae by 72 hours.

Two factors which work against the survival of fertilized eggs that have been X-irradiated are: (1) This stage is diploid (but not syngamic), in that each egg contains both male and female pronuclei, although separated. (2) In the recently fertilized egg there is movement of the sensitive elements, the chromosomes. Whether it is motility *per se* or the condition of the chromatin which is motile that is responsible for radiosensitivity would be difficult to decide, as it is in ordinary mitosis. It can easily be imagined that there are more radiosensitive elements involved when two nuclei are approaching each other than in the unfertilized haploid egg, or even after syngamy has been achieved.

Once an egg is inseminated, the dynamic processes of development normally continue without interruption. By 2¼ hours every egg was in the two-cell stage, and these were exposed to 300–1500 r of X rays. Cleavage was not stopped in any of the eggs until stages 6 to 12 were reached, depending on the X-ray dosage. Although the cleaving eggs were not killed immediately, they proved to be more radiosensitive than the uterine eggs. Four per cent gastrulated following 600 r but died thereafter, while 54% of the 300-r first cleavage stages reached the neurula stage. The few that survived for 4 days were amorphous masses. It would be of considerable morphological interest to spot irradiate one blastomere of the two-cell stage and to determine the effect on development. This would be an improvement over the standard procedure of unilateral cauterization.

The blastulae ranged from the 16-cell stage (6) to the late cleavage stage (9) and the X-ray exposures ranged from 180 to 2880 r (pl. 1). The higher dose killed all at the late blastula stage, none gastrulating. Doses of 900 r or more prevented gastrulation but did not kill the embryos for 48 hours. Exposure of 720 r allowed a few blastulae apparently to neurulate. These embryos *appeared* to be neurulae, but on histological examination, they were found to possess no central nervous system whatsoever. The notochord, gut, and oral suckers were present but poorly developed. Since 360 r did allow 20% of such blastulae to develop some semblance of a central axis, it must be assumed that a dose of 720 r to the blastula stage produces aneurogenic embryos. After 360 r the spinal cord developed abnormally and was generally occluded with loose neuroblasts sloughed off from the ependymal layer. An exposure of 180 r had very little damaging effect, and the resulting larvae lived indefinitely.

Some developed edema and a few were stunted, but many remained quite normal (pl. 1). The blastula stage must therefore be considered as extremely radiosensitive. In addition, it is of interest to point out that X irradiation at this time prevents neural development even though no cells can be identified as preneuroblasts. True, Spemann and others have mapped the late blastula for presumptive areas, but it is doubtful that even he believed the forces of differentiation were at work so early in development.

The gastrula was taken at stage 11 when the dorsal lip was crescent shaped. No dose used stopped the process of gastrulation once it was initiated, and even after 1500 r about 7% of the gastrulae appeared to be normal neurulae. Approximately 93% were living hemineurulae, indicating damage to the mechanism of incorporating the yolk: these all died by 72 hours. Following exposure to 600 r there were various degrees of development from amorphous masses to normal-appearing (stage-18) tadpoles. None of these survived, however. Following 300 r the gastrulae all developed into the external gill tadpole stage but every one exhibited some degree of ventral flexure of the tail. Some of these tadpoles lived for many days. The effect of irradiating the early gastrula stage seems to be concentrated on the posterior rather than the anterior structures at the lower doses used.

The yolk plug stage (12) given 900 r or more of X rays was not stopped in development before reaching tail bud stage (17). Most of the survivors were stunted by virtue of losing their yolk before hatching from their jelly capsules. Some edema developed, particularly in the 600-r survivors. None of the 300-r stages developed any edema, but every one had a ventral curvature of the entire body. Most of these died by 9 days. This suggests damage to the central axial structures, particularly toward the posterior where there is active proliferation during gastrulation and the yolk plug stages.

Primary neural differentiation has already been achieved by the neurula stage, X irradiation would therefore not be expected to alter axial development as it did with the earlier stages. Radiation tolerance was better; even 1500 r did not stop development until stage 19 or 20 was reached, and the tadpoles lived for some 5 or 6 days (pl. 2). At the higher dose levels, however, all developed belly edema and many acquired blisters on the dorsal aspect at the point of origin of the tail. This is the region of the transient neurenteric canal. At the lower exposures there was less edema, but there was stunting and ventral flexure of the tail. At 300 r ca. 92% of the developing tadpoles were quite normal, a few showing retardation in the development of external gills. Since some outlived the duration of the experiment, it can be said that the neurula can tolerate 300 r X rays very well.

Exposure to higher doses of X rays, e.g., 1000 r, did not immediately stop development of the organism as a whole (pl. 2). The neuroblasts, which were most active at the time of exposure, were severely damaged. Embryos surviving for 48 hours or more all showed anencephaly, with no evidence whatsoever of any brain or spinal cord. The notochord was present, although enlarged and highly vacuolated. The embryos bore a superficial resemblance to stage-20 tadpoles. Nevertheless, at no region in any of these forms could be found any neural-like tissue. Superficial suckers were present, the archenteron was structurally and topographically normal, the endo-, myo-, and pericardia and the aortic arches could all be identified, but there was no central nervous system (pl. 2). None of these embryos survived more than 5 days, but it is still of interest ontogenetically to note that the obliteration of the elements of the neural axis did not affect the early development of certain other vital structures, some of which (such as the notochord) normally appear before and others (such as the heart) after neural differentiation.

The hatching stage (19) was similarly exposed but no radiation level produced any alteration in the hatching process. Quite the contrary, radiation seemed to accelerate this process, possibly by causing a swelling of the jelly. This stage survived 300 r of X rays without detectable effects, but exposure to 600 r of X rays caused retardation in development. Fifty-four per cent survived for 5 days, during which period they all developed lordosis or a dorsal curvature of the spine. At the higher levels of exposure there was also some edema. A few hatching embryos exposed to 5000 r survived for 72 hours.

The early external gill stage (21) is very active, with both muscle and ciliary contraction. X-ray exposures of 22–15,000 r were used. Above 2000 r all such early tadpoles were dead within 4 days after exposure. Those subjected to 1000 r survived for 7 days. Tadpoles given 500 r were still alive 3 months later and were in the process of metamorphosis.

Histologically, the ciliary body of the developing eye, lens epithelium, and the mantle layer of the brain and spinal cord had tracts of pycnotic nuclei at dose levels as low as 132 r, even though these early tadpoles survived such exposure indefinitely and without any serious effects. The control and irradiated eyes showed mitoses in other regions of the retina but none where these pycnotic nuclei appeared in eyes of irradiated tadpoles (pl. 3). Pycnosis was therefore not correlated with regional mitosis. Furthermore, the number of pycnotic nuclei increased with increasing dose until at 300 r there were at least twice as many such pycnotic nuclei as after 132 r, and even more after 500 r. Other layers of the retina did not show these concentrations of pycnotic cells even at higher exposures and examination of many control eyes showed few mitoses. At this stage the muscle, cartilage, liver, gut, and even the thyroid and hypophysis show no evidence of pycnosis and are therefore presumed to be relatively radioresistant. However, the actively differentiating cells of the eye and mantle layer of the brain and cord contain many pycnotic nuclei because many neuroblasts are actively differentiating in neural pathways into neurones or neuroglia and are very radiosensitive. These are the layers which continue differentiation for a relatively long time, giving rise to connective tissue neuroglia. Radiation-induced neuroblast pycnosis did not immediately cause the death of the host. Since these cells were rarely seen in mitosis, it must be presumed that they were radiosensitive apart from the process of mitosis.

Tadpoles of stage 23 and older tadpoles of 1 and 2 months of age were also X-irradiated. Stage 23, which is in the process of absorbing its external and developing its internal gills, tolerated 1000 r and survived indefinitely. After 2000 r, 20% died within 8 days, but others lived for weeks. After 15,000 r, 50% were dead in 9 days, but their survival for that long is of interest. True, these are poikilothermic forms with low metabolic rate which might explain their slow reactions, but they were irradiated in an aqueous medium in which more deleterious and rapid effects might be expected. These tadpoles ate and digested their food, and appeared to be quite normal except for a slight stunting. About 24 hours prior to death all developed head edemas.

The 2-month-old (premetamorphic) tadpoles generally showed somewhat better tolerance of X radiation than the younger tadpoles. A few of these older stages given 6000 r were still alive 6 weeks after X irradiation.[1] The hind limbs had developed in some, the tadpoles were eating ravenously, but the metamorphic changes appeared to be retarded (pl. 4).

In embryonic stages of development the usual LD_{50} or minimum LD_{100} dose levels have at least a different meaning from the more static and uniform adult stages of the same organism. Relatively few early embryos survive even the lowest dose of X rays if an extended period, such as 30 days, is considered. On the other hand, no stages are killed outright, even with doses of a few thousand roentgens. To derive survival figures the duration of the observation would have to be varied with the stage of development irradiated, unless all periods are extended to the very critical changes at metamorphosis. It is doubtful that any embryonic stage before external gill tadpole stage 23 could, under such conditions, tolerate X irradiations much below the level of 300 r used in most of these observations. Since 132 r produced pycnotic nuclei in the ciliary body of the developing eye and the mantle layer of the brain at stage 21, this level must be far above those which would interfere with ultimate metamorphosis. Radiosensitivity of the embryo varies from stage to stage simply because different organ primordia are appearing and some are certainly more vital to the survival of the organism than are others. The embryo is a mosaic of constantly changing dynamic centers. It appears that the central nervous system has the most protracted period of differentiation of all organ systems of the developing frog embryo, thereby showing the most frequent and extensive effects of X-ray exposure (pl. 4).

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[1] At this writing, 6 weeks have elapsed since these treatments were given.

Even though lethal-dose figures cannot be determined for the embryo, X rays will prove of great importance in deriving developmental patterns in view of the hypersensitivity of differentiating cells. By irradiating the various stages, beginning with the blastula, the anlage maps that have already been derived by post-factum reasoning can be checked. X rays, by causing pycnosis, can be used to map out the differentiating tracts at any specific period of development. For example, accurate determination can be made of the time when the presumptive limb bud cells are first losing their totipotency and are being directed toward their final organ differentiation, simply by exposing successive preprimordial stages to X rays. When pycnotic nuclei are formed in the presumptive region, it can be presumed that these are differentiating cells.

Radiosensitivity of the embryo varies from stage to stage; it is not chronologically increased or decreased. This is because different organ primordia are appearing at the different stages and some are more vital to the survival of the organism than are others. The early embryo must be visualized as undergoing rapid dynamic changes with an ever-increasing mosaic of differentiations. If it is conceded that the differentiating cell is more radiosensitive than is the differentiated cell, then embryonic radiosensitivity is understood.

There is one more consideration. With each division, the potencies of the original zygote are diverted, and low exposures to ionizing radiations may damage certain cells in one blastula that are missed in another. The variation in response of individual embryos at any stage is therefore likely to be greater than among adult forms of the same species. Furthermore, if the primary damage to the embryo is the effect on chromosomes, the exposure of the earlier stages would obviously affect more of the later stages simply because, for example, approximately half of the neurula is derived from one of the first two blastomeres.

In spite of these statements, the various stages of amphibian development can be arranged in order of decreasing radiosensitivity. The most sensitive is the recently fertilized egg, the least sensitive is the tadpole. The list of developmental stages in sequence of decreasing radiosensitivities would be as follows:

1. Fertilized egg, the most sensitive stage
2. Blastula
3. First cleavage
4. Uterine egg
5. Gastrula
6. Yolk plug
7. The ovarian egg
8. The adult frog
9. The neurula
10. Stage-23 tadpole
11. Premetamorphic tadpole

DISCUSSION

The statement is often made, as it is made here, that the differentiating cell is the most radiosensitive of all cells of the living organism. This observation is based on two premises; first, with the background of experimental embryology and its spot-vital-staining or regional-injury techniques, maps of presumptive areas have been derived for those early stages when there is no morphological or cytological differentiation; second, histological examination of various embryonic stages, previously exposed to X radiation at sublethal levels, has revealed regions of high concentration of nuclei which are karyorrhectic or pycnotic. These are not regions which show a high mitotic index. These two totally unrelated methods of study point toward exactly the same regions of the undifferentiated embryo. That is, evidence from post-factum mapping of presumptive areas and the X-radiation production of pycnotic nuclei present circumstantial evidence that the differentiating cell is highly radiosensitive. Such a finding should prove to be an aid in both embryogeny and in regeneration studies. The final proof would be to excise and/or transplant control and X-irradiated groups of cells, believed to be in the process of differentiation, into different regions of irradiated and nonirradiated hosts. Until that is accomplished, there is ample reason to believe that, entirely apart from mitosis, the embryonic cell in the process of differentiating is extremely radiosensitive. And, of all the differentiating organ systems, the central nervous system and its related sense organs (particularly the eyes) are very radiosensitive over most of the period of embryonic development.

SUMMARY AND CONCLUSIONS

1. Frog tadpoles can be killed by 160,000 r or more of X radiation in a period of 1½ hours, and such tadpoles exhibit a drastic depreciation in their mucopolysaccharide reserves, particularly in the periphery of the central nervous system, the eyes, and muscles.
2. Cysteinamine extends for a short time the life of X-irradiated tadpoles receiving a lethal dose, but does not protect them against the lethal effects of exposure.
3. The newly fertilized egg and the blastula are the most radiosensitive stages in early embryonic development of the frog, whereas the ovarian egg, the neurula and the older tadpole are the most radioresistant. These differences are explained on the basis of (1) the state of the chromosomes, (2) the relative potency of the blastomeres, and (3) the presence of cells differentiating into vital organ primordia.
4. Radiation studies on the early embryo can hardly have statistical value for teratologies are not subject to quantitative evaluation. Damage following 300 r may be tolerated

at the neurula stage because vital differentiations have been accomplished. It may not be tolerated by the fertilized egg because the developmental potencies of the entire organism are not yet segregated and differentiated.

5. Low-dose X irradiations may prove of value in mapping developmental potencies at an earlier stage than has been hitherto possible, simply because of the hypersensitivity of the actively differentiating cells. These cells cannot be identified morphologically, but they may be located radiocytologically.

6. The differentiating cell, even prior to its mitosis, is more radiosensitive than is the differentiated cell in the process of mitosis.

7. There is a latent period between exposure to X radiation and the cessation of cleavage, or the appearance of developmental abnormalities. Pycnosis and karyorrhexis may be caused immediately, however. Development is never stopped precipitously, in spite of cellular damage.

8. The most frequent types of developmental abnormalities which are seen in embryos previously exposed to ionizing radiations are:

1. Pycnosis or karyorrhexis of differentiating cells
2. Exogastrulation
3. Edema
4. Flexure
5. Aneurogenic development
6. Amorphous development

9. It can be said that any embryonic stage (excepting the tadpole) is infinitely more radiosensitive than the adult of the same species, by any criterion of estimation. This may be related to the protracted period of differentiation of the central nervous system and its accessories.

DISCUSSION

Brunst: I have an impression from some of your pictures that the rods and cones layer in some instances disappear from the retina. Did you observe here something similar to the giant cells in irradiated tissues described in some of your previous papers?

RUGH: There are giant cells in practically all necrotic radiation-damaged tissue — cells of all different sizes, abnormal cells. The rods and cones are separated from the pigmented layer, but are not individually damaged as are the neuroblast cells, what we call the formative cells, or the embryonic cells in the retinal layer. The rods and cones are apparently not relatively sensitive or delicate. Edema develops and separates the rods and cones from the outer pigmented layer. But I do not believe the cells of this layer are as easily damaged as other cells. Since those later tadpoles given a low dose and having neuroblast cells in the margin of the eye do survive, it would be of interest to carry them along to the postmetamorphic stage and see if there is any residual effect.

I happen to be of the school of experimental radiologists who cannot conceive of recovery from radiation damage to a nucleus which results in the pycnotic change. It is hard to imagine that, once achieved, the pycnotic condition can ever be reverted to the normal condition. There may be replacements.

HICKS: Many of those cells that Dr. Rugh showed are plagiocized and carried away. They are completely dissolved.

HAYMAKER: We think most of those things are reversible.

RUGH: Maybe we should specify that we are discussing only radiation-produced pycnosis.

GRUENWALD: If this pycnosis is similar to what I have seen, for instance, in the effects of selenium on chick embryos, it is really not pycnosis. It is fragmentation of nuclei and therefore indicates cell death.

I should like to bring up another point of terminology. The term ''anencephaly'' has been used to designate something else for many years. I wonder whether it would not be a better idea to use a different term. In anencephaly the neural plate has remained open but the brain tissue was originally there. I am afraid some confusion will result if some medical people read in your papers that you produced anencephaly.

SCHJEIDE: If the tadpole some months of age may be considered an embryo, or at least not an adult, it may be pertinent to relay some information which has resulted from the investigations of Dr. Allen, myself, and others at the UCLA Atomic Energy Project. First of all I wish to state that leucopoiesis in the tadpole is almost totally stopped for a period lasting at least 25 days by a dose of 250 r of X radiation. These cells are not inhibited from dividing; in fact, they continue to divide for approximately 48 hours, but as each cell attempts to divide it breaks down. This process results in a gross depletion of the hematopoietic tissues. It is not positively known whether sensitivity of the leucopoietic cells differs in the tadpole and the adult frog because no parallel experiments have been performed on the adult. The indications are, however, that the adult hematopoietic cells would prove equally sensitive. Also, the pronounced effect of 250 r on the leucopoietic tissues of both birds and mammals, adult and embryonic, would seem to point toward the notion that leucopoietic tissues are physiologically similar in a wide variety of animals and at different stages in the same animal.

Our work on tadpole hematopoietic tissues has shown that, in these cells, the rate of division (which can be controlled in various ways) determines the extent to which the tissues are damaged. It may be possible to relate this observation to the reproducible abnormalities which have occurred in the amphibian and mammalian embryos after doses of radiation. It may be that the region in which cell division is most active at the time of irradiation is slowed in development because of the increased breakdown of dividing cells in this region. The abnormalities which follow may be the result of lack of sufficient inductor tissue, lack of some necessary secretion or component of the blood, or they may be purely mechanical deformation.

A word might also be said in regard to the dependence of the amphibian embryo on environmental conditions, especially temperature. Since it has been shown that lower temperatures retard the division rate in amphibians and since cell destruction may take place more rapidly where the cells are undergoing frequent mitosis, it seems possible that various abnormalities following irradiation may be modified in extent, at least as observed within a given time, by changes in the environmental temperature.

Finally, Dr. Rugh has mentioned the work which we have done with the amphibian pituitary. We must emphasize that thus far we have used pituitaries from recently metamorphosed frogs. Still, there is reason to believe that this pituitary is embryonic in nature: it is at least growing quite rapidly at this stage. We have found that, even with a dose of γ radiation as high as 200,000 r there is active formation and secretion of protein hormone for the short time that the pituitary cells remain intact. This would point to the view that some of the metabolic processes are very resistant to radiation, and it further serves to caution somewhat against the view that in the irradiated embryo abnormalities and retardation are caused by effect on either protein synthesis or secretion mechanisms *per se*.

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PLATE 1

EXPLANATION OF FIGURES

A. X irradiation of the frog blastula; stage 8.

1–4 All at 3 days. 1, control; 2, 180 r, slight stunting; 3, 360 r, failure of head differentiation; 4, 720 r, died as exogastrula.

5–7 All at 4 days. 5, control; 6, 180 r, slight stunting, retardation of anterior structures; 7, 360 r, failure of neural derivatives.

8–10 All at 5 days. 8, control; 9, 180 r, slight retardation; 10, 360 r, edema, stunting, poor differentiation.

B. Histological study of frog embryo 1 and 3 days after exposure of the blastula stage (7) to X rays of 180–720 r.

11–13 Rather normal development of eye and central nervous system following 180 r of X rays.

14 Liberation of neuroblast cells into the myelocoele.

15 Disorganization of the metencephalon.

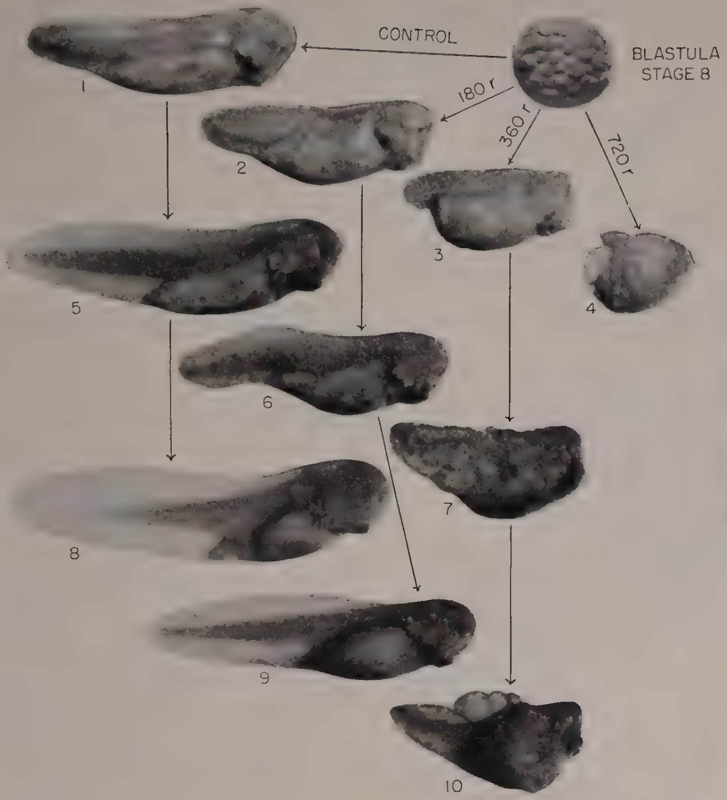
16 Presence of pronephric tubules but shrinkage of yolk.

17 Complete dispersal of the presumptive spinal cord cells.

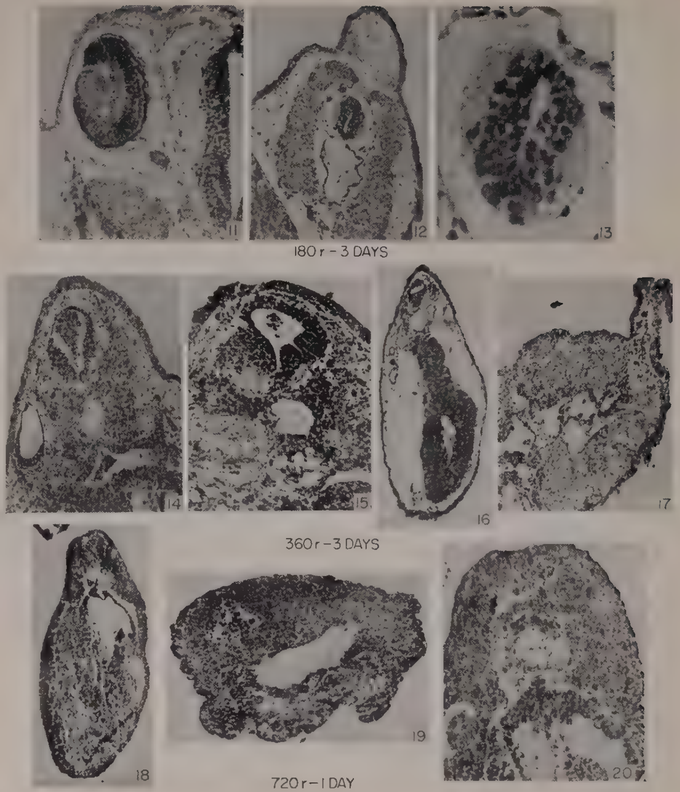
18 One day following 720 r of X rays the ''neurula'' stage is without any nerve cord, although the archenteron and notochord are present.

19 The suckers and gut appear, but there is no brain or anterior notochord.

20 At mid-body level only the notochord is clearly recognizable.



A



Blastula stage 7
B

PLATE 2

EXPLANATION OF FIGURES

A. Effect on embryonic development of X irradiation of the neurula stage. Note vesicles at region of transient neurenteric canal following exposure to 600 r or more; stunting at 900 r and belly edema at 1200 r.

B. Histopathology of the embryo following X irradiation of the neurula.

1 Cross section at the level of the suckers showing anterior tip of the notochord but complete absence of brain and foregut.

2 Enlarged view of region where the developing brain should be.

3 Section at level of pharynx.

4 Section at level of aortic arches.

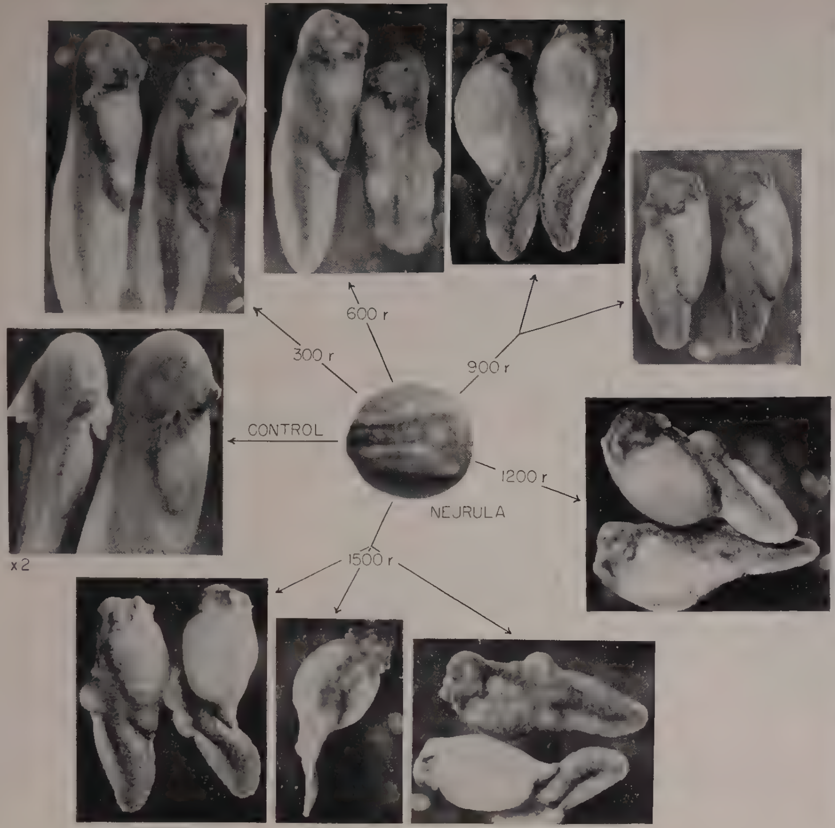
5–6 Sections at level of developing heart. Note endo-, myo-, and pericardia, and gill bulges.

7–8 Enlarged view from figure 9 to show rather normal notochord but complete failure of neural differentiation. Identify endo-, myo-, and pericardia.

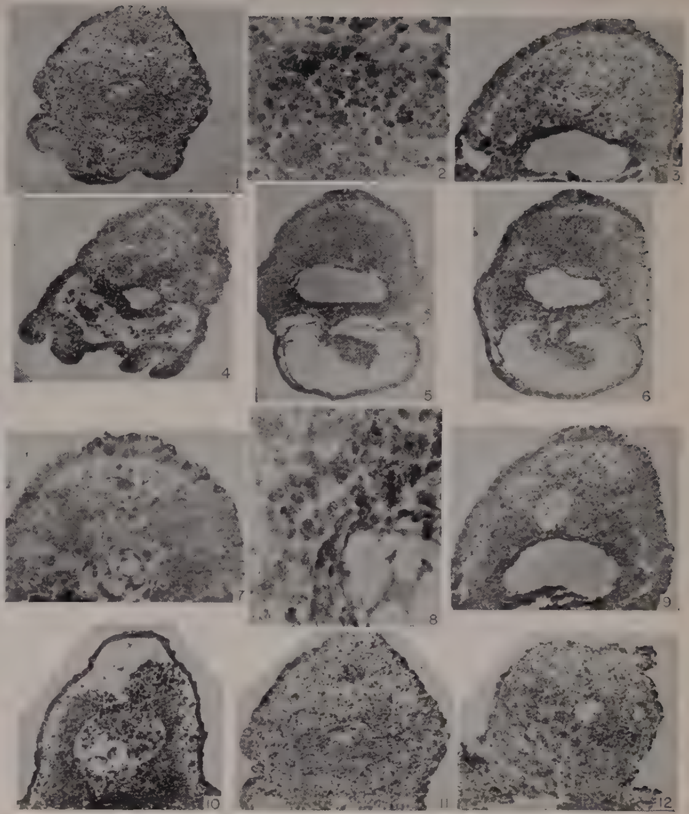
9 Section through level of midgut showing large notochord but no developing spinal cord.

10 Section at mid-body level. Note enlarged and vacuolated notochord.

11 12 Sections at posterior levels showing dorsal tail fin, still with no neural differentiation.



2 days postirradiation
A



Early neurula stage 13, 1000 r, 2 days
B

PLATE 3

EXPLANATION OF FIGURES

A. Effects of X irradiation of the external gill, stage 21.

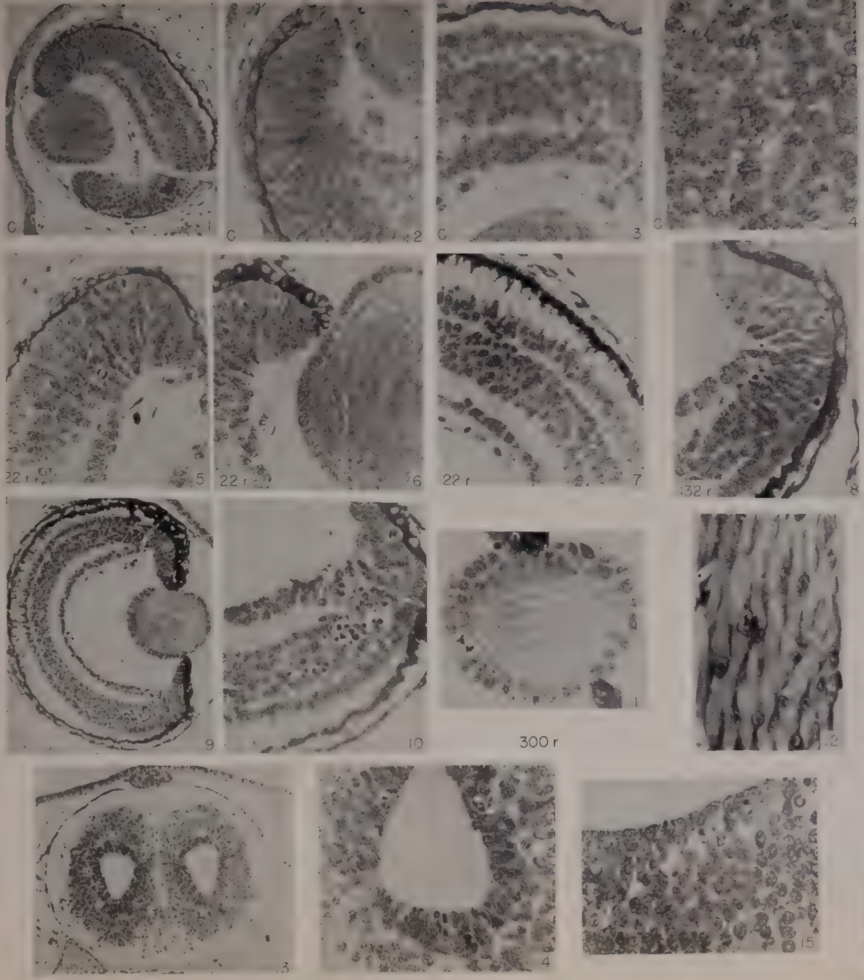
Sections of the control at the time of exposure.
1 Developing eye showing normal mitoses.
2 Enlarged view of margin of optic cup.
3 Enlarged view of retinal layers.
4 Enlarged view of brain (telencephalon).

Following exposure to 22 r of X rays.
5–6 Margin of eye. Note normal mitoses.
7 Retinal layer of eye, showing only slight (edema) separation of layers by X rays.
8 Following exposure of 132 r of X rays. Note some normal mitosis (marginal) and a few pycnotic nuclei in region of developing ciliary body.

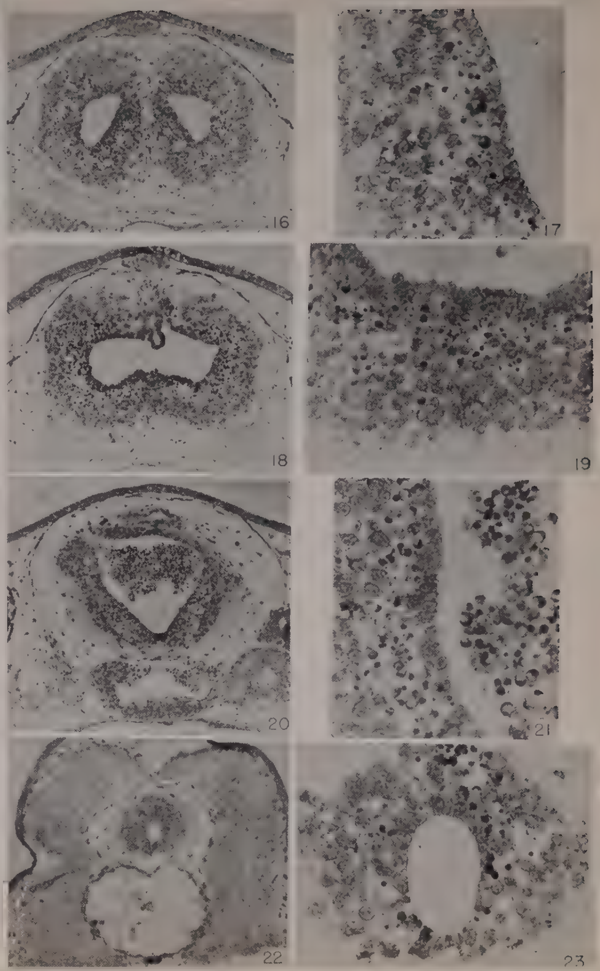
Following exposure of 300 r of X rays.
9–10 Eye, showing more pycnosis.
11 Lens epithelium showing abnormal mitosis.
12 Muscle bundle without pycnosis.
13 Telocoels at level of epiphysis, with scattered pycnosis in telencephalon.
14–15 Enlarged view of telencephalon, showing both mitosis and pycnosis.

B. Effect of 6000 r of X rays on the developing central nervous system of the amphibian embryo.
16–17 Telencephalon.
18–19 Brain at level of epiphysis.
20–21 Brain at level of hypophysis.
22–23 Spinal cord at mid-body level.

Note abundant pycnotic cells among otherwise apparently normal (differentiated) nerve cells of the central nervous system. Many damaged (neuroblast) cells liberated into the embryonic brain cavity.



External gill stage 21
A



Developing central nervous system; 6000 r, 1 day
B

PLATE 4

EXPLANATION OF FIGURES

A. Effect of 500 r of X rays after 2 days on the premetamorphic (2-month-old) tadpole.

Controls
1 Lymph node.
3 Telencephalon.
5 Margin of eye.

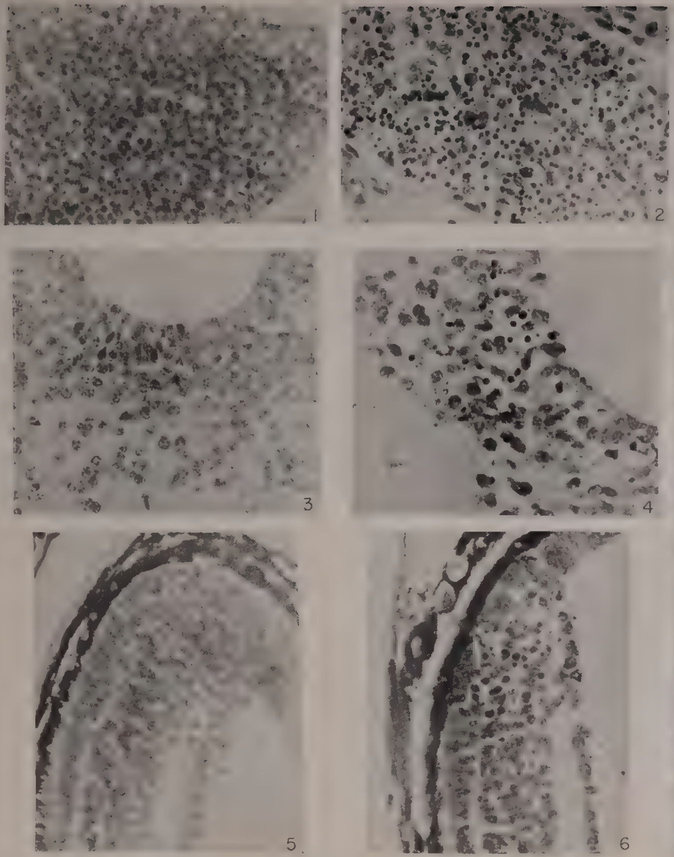
X-irradiated
2 Lymph node, extensive damage.
4 Telencephalon, pycnosis and dying cells.
6 Margin of eye, damage in form of pycnosis restricted to region of active differentiation.

In spite of the illustrated damage, these tadpoles live indefinitely and achieve metamorphosis normally.

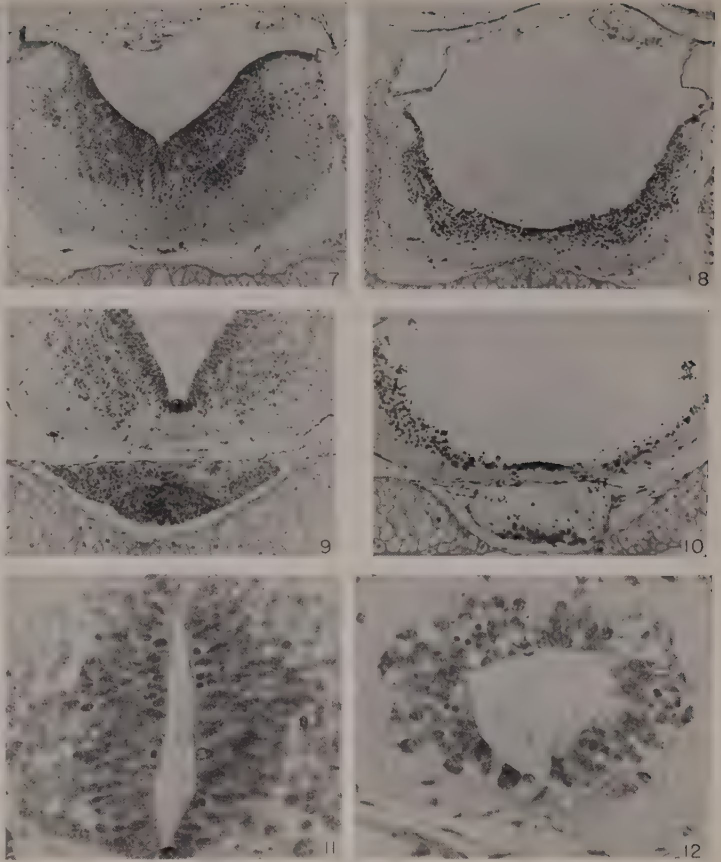
B. Effect of 42,000 r after 6 days on premetamorphic (2-month-old) tadpoles.
7 Controls, level of diencephalon.
8 X-irradiated, level of diencephalon.
9 Controls, level of pituitary body.
10 X-irradiated, level of pituitary body.
11 Controls, level of spinal cord.
12 X-irradiated, level of spinal cord.

Demonstrating the susceptibility of the central nervous system throughout embryonic (tadpole) life, while in the same animals the differentiated (muscle, cartilage, etc.) tissues are apparently undamaged.

ROBERTS RUGH



Premetamorphic tadpoles, 500 r, 2 days
A



Premetamorphic tadpoles, 42,000 r, 6 days
B

RADIOTRACER TECHNIQUES IN EMBRYOLOGICAL RESEARCH

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SEVEN FIGURES

Although radioisotopes have come to be used almost routinely in certain clinical procedures and in certain types of physiological research, their application in developmental physiology is still, generally speaking, at the exploratory stage. However, radiotracers provide a means for obtaining biochemical information at the cellular, even the molecular or atomic, level and it is to be expected that the passage of time will bring about refinements of present-day tracer techniques, a general awareness of their potentialities, and, subsequently, their widespread application in the study of problems of development. Meanwhile, let us examine the phases of developmental research in which radioisotopes have already been put to use.

CHEMICAL DIFFERENTIATION OF EGGS AND SPERM

Among the first applications of radioisotopes in biology was the use of radiophosphorus by Hevesy and Hahn in 1938 in a study of the origin and deposition of materials in the hen's egg. They administered labeled sodium phosphate to laying hens and then measured the radioactivities of extracted ovarian, plasma, and liver phospholipids. From their results they concluded that the phospholipids of the yolk are synthesized in the liver and carried by the plasma to the ovary. Although nonphosphatide phosphorus (mainly in inorganic form) was deposited in yolk, albumin, and shell at all stages of development when eggs from untreated hens were immersed in solutions of radiophosphorus, the yolk phosphatides showed little radioactivity. From these data it was further concluded that there is no new formation of yolk phosphatides after the egg leaves the ovary.

Lorenz *et al.* ('42), working along similar lines, have demonstrated that when P^{32} is administered during shell formation, it very rapidly enters the shell but that its incorporation into the albumin takes place after a delay of several hours. Thus the phosphoproteins in the albumin are formed during the early stages in the growth of the yolk and are ready for deposition around the yolk as it moves down the oviduct.

The incorporation of calcium into the hen's egg has been studied by Spinks *et al.* ('49) and by Comar and Driggers ('49). Both of these teams reported that after Ca^{45} was administered to hens an appreciable amount was located in the shell of the first egg laid after injection (only 15 minutes after administration in one instance). Comar and Driggers found that in the second egg laid the radioactivity of the white was seventy times that of the yolk but that in later eggs the yolk eventually became more radioactive than the white, adding weight to the conclusion of Lorenz *et al.* ('42) to the effect that the white and yolk of a given egg are synthesized almost simultaneously.

Recently, Odeblad ('52) prepared autoradiograms of ova from rabbits which had been injected with S^{35}-labeled sodium sulfate. He observed that the sulfur uptake of the egg itself was relatively low as compared with the granulosa, and concluded that the sulfate ion is not utilized in the synthesis of amino acids within the egg.

The uptake of P^{32} in Tyrode's solution by bull sperm has been shown (Bishop and Weinstock, '48) to vary directly with the motility of the sperm. More than 75% of the phosphorus which penetrated the cells persisted in the form of free inorganic phosphate. Both anaerobic conditions and an excess of glucose in the medium increased the rate of uptake (Bishop, '50). Fuller investigation of these findings may uncover some very interesting information regarding the energetics of sperm movement.

MOVEMENT OF MATERIALS INTO, FROM, AND WITHIN DEVELOPING INDIVIDUALS

Embryos and larvae which develop in marine or fresh water. Materials are exchanged directly between the embryo and the environment among those invertebrates and lower vetrebrates which develop in marine or fresh water. A considerable body of literature has been accumulated concerning this sort of transfer in the embryos of marine invertebrates, particularly the sea urchin. The application of radioisotopes in this field was first reported by Abelson in 1947, who made measurements of the permeability of sea urchin embryos to radiophosphorus by a basically simple technique. The embryos were immersed in radioactive solutions under controlled conditions, removed, and washed. Measurements of their subsequent radioactivity then gave values from which the P^{32} uptake was calculated. In a similar fashion the rates of entrance of potassium (Chambers *et al.*, '48; Chambers, '49) and phosphorus (Whitley, '49) have been investigated. All these authors have been concerned with the changes wrought in the egg at fertilization: in each instance the uptake rate for the elements listed has been markedly greater in fertilized than in unfertilized eggs. These studies on the sea urchin embryo are particularly important because of the rich background of correlative biochemical and biophysical information which has been built up concerning that form; however, there is a need for their continuation and for similar studies on other forms.

Interchange of materials between the environment and the aquatic larva has received some consideration. Hassett and Jenkins ('51), who were interested primarily in the effects which its radiations would produce, measured the uptake of P^{32} in mosquito larvae. They discovered that phosphorus readily entered larvae up to the fourth instar, at which stage the larvae cease to feed. After that stage they took up little. Phosphorus was apparently taken up primarily through the gut wall but also through the anal gills and, perhaps, through the integument.

In a preliminary study of iodine turnover in larvae of the tree frog, *Hyla versicolor,* Dent and Hunt ('53) found that, over a temperature range which approached the limits of viability for that form (10°–31°C.), the rate of uptake was greater at the higher temperature than at the lower and that the uptake rate increased as the iodine concentration of the medium was increased up to 1000 parts per million (the highest concentration tested). However, these same variations in temperature and iodine content of the surrounding medium had no appreciable effect on the rate at which iodine was eliminated from the tadpoles. Approximately 50% of the iodine retained after 24 hours of immersion in a radioactive solution was consistently released within 24 hours following removal from that solution, 70% within 48 hours, and 85–90% within 5 days.

Avian embryos. Exchange of materials between the embryo of the bird and the yolk or white of the egg appears to be somewhat more complex than that which obtains for the aquatic embryo and its immediate environment. At least, this is the indication of the results of Wolken ('50) and Epstein and Wolken ('51) who showed that, when a solution containing inorganic radiophosphorus was injected into the white of a hen's egg, the phosphorus became evenly distributed throughout the white within a period too brief to account for movement by simple diffusion so that movement by convection was postulated. These authors have derived mathematical equations which describe the process as it takes place during early development, but a new, and as yet unexplained, situation develops with the onset of a period of rapid growth beginning on about the fourth day of incubation. As was observed in the sea urchin and the frog (Abelson and Duryee, '48), fertilization has a marked effect on ionic transport and the transport rates for P^{32} are much greater in fertile than in infertile hens' eggs.

Fetal and newborn mammals. Prior to 1939 numerous studies were made concerning the transport of materials across the mammalian placenta, but there remained a lack of quantitative data on this subject. In that year there appeared the first of a series of papers (Flexner and Roberts, '39) by Flexner and his coworkers describing experiments in which the transfer of radioactive sodium was studied in the placentas of several different mammals. The accumulated information obtained by this group was summarized in 1942 by Flexner and Gellhorn with the general conclusions that: (1) the transfer rate of physiological substances across the placenta increases throughout the gestation period until just before term when a sharp decrease occurs; (2) variations in transfer rates among the various mammals are correlated with placental morphology, i.e., the smaller the number of layers between the maternal and fetal blood streams, the greater the transfer rate per unit weight of placenta; and (3) in general the rate of transfer of a given substance parallels the relative growth rate of the fetus.

Following the beginning made by the Flexner group, Pommerenke *et al.* ('42) presented the initial publication on the transfer of radioactive iron across the human placenta. The use of this tracer element very quickly produced evidence indicating the maternal plasma to be the primary source of fetal iron. Reinforcing evidence came from the work of Vosburgh and Flexner on the guinea pig in 1950. Using mice, rats, or guinea pigs, the movement of calcium, strontium, plutonium, and copper through the placenta have been followed by radiotracer techniques (Pecher and Pecher, '41; Finkel, '47; Schubert *et al.*, '48).

After parturition the delivery of materials to the newborn mammal in milk from the mother comes to be of interest. This is another phase of developmental physiology attacked by means of radioisotopes shortly after they first became available. In 1938 Aten and Hevesy injected lactating goats with radiophosphorus in the form of sodium phosphate and found that the radiophosphorus had been incorporated in various constituents of the milk within a few hours following injection. Radioisotopes were then used to measure the rates of secretion for strontium (Erf and Pecher, '40) and phosphorus (Kleiber *et al.,* '48) in cow's milk. Likewise, the lacteal transmission of iron, calcium, strontium, plutonium, and iodine has since been studied in rats and mice by means of radioisotopes of those elements (Pecher and Pecher, '41; Finkel, '47; Huggett and Widdas, '49; Rugh, '51). Since earlier methods had produced conflicting findings concerning the course of blood flow in the fetal heart, Everett and Johnson in 1950 devised and used a radiotracer technique in investigating this phenomenon. Fetal guinea pigs and dogs were delivered by Caesarian section in such fashion that the placental circulation remained intact. After radiophosphorus had been injected into the umbilical and external jugular veins, the buildup of radioactivity was measured in both ventricles, and from the data obtained the course of the blood through the heart was clearly charted.

DISTRIBUTIONAL PATTERNS OF VARIOUS ELEMENTS

The ascertaining of the distributions of various elements in animals at various stages of development is a type of pioneering study which has received the attentions of several workers. Autoradiographic rather than counting techniques have usually been employed in these investigations because of the difficulties involved in dissecting and handling small portions of embryonic or larval tissue and because the autoradiogram permits one to locate more precisely regional variations in relative concentration of radioisotopes within minute organs or organ rudiments.

Phosphorus and copper. The distribution of radiophosphorus has thus been traced in the larvae of wax moths and mealworms (Lindsay and Craig, '42), in larvae and pupae of fruit flies (Irwin *et al.,* '50), in chick embryos (Hunt and Wolken, '48), in the embryos and young larvae of frogs (Hansborough and Denny, '51), and in metamorphosing frog larvae (Gennaro and Dent, '51). In a similar manner Smith and Gray ('48) have followed the distribution of Cu^{64} in developing chick embryos. The common finding of all these investigations is that phosphorus and copper tend to become localized in those regions in which growth and/or differentiation are progressing most rapidly, as shown in figures 1, 2, and 3 (pl. 1). This is not surprising when one considers the several ways in which phosphorus interacts in the metabolism of growth and differentiation and the importance of copper in the cytochrome system. These studies are limited in that they do not indicate the chemical character of the compounds with which the radioelements under observation are linked at the sites of concentration; however, as Needham ('31) has said, "A remarkable survey of wide tracts of the mechanism of the egg can be obtained simply by observing where the phosphorus is (concentrated)" and such studies do pave the way for future studies of a more quantitative nature.

Iodine. The distributional patterns of radioiodine, on the other hand, are somewhat more specialized. Wheeler ('50) prepared autoradiograms of I^{131}-treated third instar larvae and pupae of *Drosophila gibberosa* and discovered an extremely high concentration in a posteroventral region of the larva where black pigment formation also occurred. In addition, she also showed localizations in larval skeletal parts and in tanned larval structures. It is of interest that Gennaro (personal communication, unpublished) observed the principal localizations of radioiodine in developing individuals of *Artemia* to be in chitinous structures and that Dent and Hunt ('52) found relatively high concentrations of I^{131} in the horny beaks and teeth of tadpoles. No ready explanation for this association of iodine with chitinous and cornified material has been offered.

In the investigation just cited Dent and Hunt prepared autoradiograms of serially sectioned specimens of *Hyla versicolor* at various larval and metamorphic stages. In addition to the localizations in the horny beaks and teeth others were seen in the thyroid, the thymus, the gut, the skin, and pigmented tissues, particularly the tapetum nigrum (figs. 4–7). The concentration in the thyroid, which was by far the most dense, needs no comment. On the other hand, the iodine uptake of the thymus is rather puzzling since there has been no previous report of active iodine metabolism in that structure. Presumably, the iodine found in the gut and skin was in the process of being excreted.

The concentration of iodine in melanogenetic regions may be a generally occurring phenomenon since, as has been pointed out earlier, Wheeler ('50) reported a heavy iodine accumulation in a melanin-forming region of the fruit fly larva and Hunt ('53) observed that, after the twelfth day of incubation, radioiodine becomes localized in the eye of the chick. Also, the present authors (unpublished) have found that iodine is fixed in the retinas of immature black (but not white) mice. Since they failed to find such fixation in the eyes of adult frogs there is some reason to believe that iodine fixation is a property of tissue in which melanin formation is actively taking place. It should be pointed out, however, that Bruner (personal communication, unpublished) was able to detect no marked localization of radioiodine in human melanosarcomas. The available information on melanogenesis leads one to suspect that the iodine localized in melanin-forming regions becomes joined to tyrosine or tyrosine derivatives to produce iodinated compounds identical with or similar to those synthesized in the the thyroid. This supposition could probably be tested by paper partition chromatography.

Barium and lanthanum. The uptake and distribution of Ba^{140} and La^{140} in larvae of *Drosophila repleta* has been examined by Bowen ('51). The radiolanthanum was neither taken up nor excreted when formed in the body by the decay of the radiobarium. The nature of the distributional pattern of the barium indicated that it represented merely functional localizations of dissolved, as opposed to particulate, food and not metabolic localization of a specific ion or ions.

UTILIZATION OF MATERIALS WITHIN VARIOUS TISSUES

The two preceding sections of this paper have dealt first with the transport of ions to, from, and within the developing individual and then, second, with the generalized distribution of certain isotopes (including some speculation as to their utilization in regions of greatest concentration). These sorts of information are of undeniable value, but it is probably of even greater importance to ascertain the precise functions and biochemical actions of the elements which are transported and distributed. This section, then, will review some studies in which specific embryonic regions or metabolisms have been examined by means of radioisotopes.

Thyroid metabolism. Since the thyroid gland is a controlling factor in the later stages of vertebrate development, it is desirable to know just when it begins to secrete its hormone. Presumably, thyroid function begins shortly after the thyroid commences to store iodine, and radioiodine has been employed to ascertain the developmental stage at which initial storage takes place in the frog (Gorbman and Evans, '41), the chick (Hansborough and Kahn, '51; Hunt and Huff, '52), the rat (Gorbman and Evans, '43), the rabbit (Jost *et al.,* '49), the hamster (Hansborough and Seay, '51), and the human being (Chapman *et al.,* '48).

The question of whether or not the endostyle is homologous to the thyroid has long been of interest to the comparative embryologist. Gorbman and Creaser ('42) have, by means of autoradiograms, demonstrated that certain endostylic cells of the larval lamprey store radioiodine, but point out that Gorbman found no evidence of iodine-storing capacity in the endostyles of four different protochordates.

Since it had been established earlier that implantation of thyroid glands in various regions of amphibian larvae elicited local metamorphic responses in neighboring host tissues, Kaltenbach ('50) implanted thyroid glands from animals which had been treated with I^{131}. She reported that, when the grafts "took" and marked metamorphic changes ensued, the iodine remained concentrated in the graft area; but that, when grafts were phagocytized and produced only slight changes, iodine diffused rapidly from them.

Phosphorus metabolism. Because of the extreme importance of phosphorus in biological systems and the present ready availability of P^{32}, the study of various phases of phosphorus metabolism is currently one of the most active fields of tracer research. Although adult material has been used primarily in such studies, embryonic material has not been entirely neglected.

Several investigations (Hevesy *et al.*, '38; Spinks, Lee, and O'Neil, '49; Hansborough and Nicholas, '49; Branson *et al.*, '50) which have involved the injection of P^{32} or P^{32}-labeled compounds into hens' eggs and the subsequent carrying out of analytical procedures have produced the generalized conclusion that the embryo, in synthesizing phosphorus compounds, first uses the small amounts of inorganic phosphorus available to it and that, later on, phosphorus is obtained from organic compounds of the yolk which are hydrolyzed outside the embryo before utilization. Similarly, Nielson ('42) and Popjak ('47) have used P^{32} in investigations from which it was concluded that the rat placenta does not transmit phospholipids and that all fetal phospholipids are synthesized within fetal tissue, at least to the point that the phosphorylating step in synthesis occurs only in fetal tissue.

There is currently expressed among many embryologists a profound interest in nucleic acids and nucleoproteins, stimulated primarily by the findings of Brachet and his coworkers ('50). Knowledge in this field is being expanded through investigations such as those of Villee *et al.* ('49), Villee and Villee ('52), and Kutsky ('50). Villee and his colleagues performed analyses on sea urchin embryos which had been treated with radiophosphorus and demonstrated that, whereas the ribonucleic acid (RNA) content during the first 72 hours of development remained constant, deoxyribonucleic acid (DNA) was synthesized, but not from the RNA present before fertilization and that, indeed, an RNA precursor was unlikely. Kutsky conducted a fractionation study on zygotes, early gastrulae, middle neurulae, and tail bud stages of *Rana pipiens* into which P^{32} had been introduced. Her results indicate a correlation between morphogenetic activity and the increased turnover of RNA, which she found to occur just after the beginning of gastrulation.

Metabolic changes at gastrulation. During the period of gastrulation, embryos, particularly those of invertebrates and lower vertebrates, undergo rapid and extensive changes in form. It is to be expected that these morphological changes are accompanied by equally pronounced metabolic alterations. From time to time evidences supporting this hypothesis have been advanced (Needham, '42; Boell, '48). A further contribution of this nature is seen in the work of Kutsky ('50). Also worthy of mention is the investigation of Friedberg and Eakin ('49) in which C^{14}-labeled glycine was applied in a study of protein metabolism in embryos of the tree frog, *Hyla regilla*. They showed that the uptake of the labeled glycine was considerable during gastrulation but negligible after neurulation.

MISCELLANEOUS PROBLEMS

The proclivity of rapidly growing tissue for the accumulation of phosphorus was pointed out earlier in this paper. Hull and Kirk ('50) have produced evidence to show that the rate of radiophosphorus incorporation in embryonic heart tissue grown *in vitro* parallels the mitotic coefficient of such tissue. The possibility of utilizing the phosphorus uptake as an index in growth measurement was explained by Stewart and Kirk in 1952. They measured simultaneously increases in area, nitrogen content, pH of used media, and P^{32} uptake in embryonic heart tissue grown *in vitro* in eight different media and found that the P^{32} uptake was, in most instances, quite reliable as the sole basis of growth measurement. However, anomalous results were obtained with two types of media, indicating that conditions must be standardized when P^{32} uptake is used as an index of growth.

The identification of engrafted tissues is occasionally difficult. Vital stains have been used to good effect in marking grafts, but diffusion of such stains from the cells of the graft, especially in chick material, occasionally makes that mode of marking unreliable. Abercrombie and Causey ('50) transplanted tissues from chick embryos which had been treated with P^{32}. Autoradiograms of the embryos which received the transplants showed good delimitation of the graft area, but the authors were unable to distinguish definitely between the individual cells of the graft and those of the host in regions where the two were mixed. With modification (e.g., using a radioisotope of some element less readily diffusible than phosphorus) this technique might prove to be quite valuable.

GENERAL CONSIDERATIONS

The difficulties to be encountered and the advantages to be gained in the use of radiotracer techniques have been amply dealt with in numerous review papers written during the past 5 years (see, e.g., Gorbman, '48; Hevesy, '48; Sacks, '48; Paneth, '49; Bourne, '52; Villee, '52). For the most part, the information cited in these reviews is equally applicable to investigations concerned with either adult or embryonic material. It is obvious that, if the radiation tolerance of cells under study is exceeded, one observes not the responses of normal cells but of cells reacting to radiation damage; but this has not yet proved to be a serious handicap in tracer research. Isotopes of a given element vary somewhat in their chemical properties [the so-called "isotope effect" (Ropp, '52)] but these variations are usually within the range of experimental error. By merely tracing and locating a radioelement introduced into an organism one does not identify the molecules with which it combines and there is no guarantee that the radioactive portion of a labeled compound will not split away before it reaches its site of localization. These difficulties must be met with by combining tracer techniques with other techniques of biochemistry. Fortunately, the way of the developmental physiologist is in this regard being rapidly paved. In his recent review paper Villee ('52) cites 249 papers which appeared during 1950 dealing with the use of radiotracers in biochemistry and medicine. This gives some idea of the fund of information which may be drawn upon by the embryologist as he begins to apply radioisotopes more extensively in his own field of investigation.

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DISCUSSION

Chairman Hamburger: Of course the experimental embryologist hopes very much that the tracer technique may help to provide an answer to one of his unresolved problems — that of embryonic induction. Since it has not yet been established that substances actually are transferred from inductors to the induced structures, it can be realized how urgently such information is needed. At any rate, as soon as the techniques are available, their combination with transplantation techniques seems very hopeful.

Brauer: In regard to the speaker's statements that morphogenetic changes are accompanied by the metabolic changes shown in these experiments — is there any evidence that metabolic changes such as deposition and transfer of isotopes precede the actual visible morphogenetic changes? And has he any evidence that there is a differential deposition of isotopes in the egg prior to fertilization?

Dent: I can only cite some rather fragmentary work which was done by Dr. Gennaro and myself but which was not followed through in detail. You noted the localization of phosphorus in that rather well-developed limb bud (fig. 1). There was indication in autoradiograms of younger animals that localized phosphorus accumulation does precede the actual formation of the limb bud.

Brauer: What is the pattern of deposition in sea urchin and in other embryos?

Dent: I do not believe that a very definite pattern was worked out in very young embryos. As I recall, there was one autoradiogram published which indicated that deposition would be heavier in the cortical than in the internal region, but that might have been simply a diffusion phenomenon. Attempts at treating frog's eggs with radiophosphorus show very poor penetration in the early embryos. However, the material administered to the *mother* passed quite freely into eggs developing within the ovary.

WEISS: Did you say that you can see phosphorus accumulation before there is any visible evidence of the limb bud? Is not that semantics? What stage of the limb bud are you talking about?

DENT: In this fragmentary observation made on one or two specimens of young larvae Dr. Gennaro and I found an accumulation of phosphorus in the region where the limb bud should eventually form.

GENNARO: I should like to add that we observed only the P^{32} localization and compared that to the morphological structure as we could see it. Possibly histochemical analysis of the tissue at the time would show chemical changes occurring, but as far as we could observe, there were no definite structural patterns yet apparent in the area observed. This was the site of bud formation still within the body of the animal. There was no protruding limb.

BISHOP: Dr. Dent, do you have any evidence for radiation damage resulting from accumulation of isotopes in the system during your tracer studies?

DENT: In carrying out a tracer experiment without any previous information concerning dosage tolerance, it is necessary to proceed empirically. There was evidence of cellular damage in some areas when higher doses of radioactive material (I^{131}) were administered to the tadpoles which we used.

The thymus, for example, which appears to accumulate iodine quite readily, was also quite susceptible to radiation damage at higher concentrations. There may well have been some damage in the eye region also, but that has not been checked thoroughly. The thyroids which accumulated the higher concentrations of I^{131} were also, in some instances, destroyed.

SHEPPARD: This work recalls some questions in biological systems which arise when the method of isotopic labeling is used.

Some of the things which have bothered me have been summarized in a paper which Dr. Householder and I (Sheppard and Householder, '51) wrote. I think I would like to amplify on one phase of this at the moment.

As we know, there have been a very limited number of experiments done with radioactive isotopes in which an attempt was made to measure precisely the rates at which things move in a biological system. The difficulty lies in our inability to follow the individual labeled atom in the system. We are thus compelled to infer the movement of substances in a system by equilibrating the system for varying lengths of time and then, at various time intervals, isolating portions for analysis. From the results we then decide how much has moved from one portion of the system to another.

A typical example of the most elementary type of such a study would be the movement of radioactive potassium between the red cells and the plasma of blood. It is true that, in studying blood *in vitro,* if the vessel is shaken, the plasma isolated for assay would be uniformly mixed, and thus homogenous. However, only if the individual exchange rates of the individual erythrocytes, i.e., the rate at which potassium is exchanged between individual erythrocytes and the plasma, were the same among all the cells, would it be possible to assume that the cellular compartment is homogeneous.

In general, most of the calculations on which we base transport measurements of that sort must of necessity assume uniform mixing, but the establishment of homogeneous compartments in most biological experiments is quite difficult.

Turning to another point, there is a definite need for us to be able to determine the transport of substances in systems where the composition varies uniformly. In such a system there would be no uniformly mixed compartment, but rather a variation in the chemical concentration of, say, phosphorus or iodine. It turns out that the movement of the isotope obeys an equation which is very similar to the Fick law equation of diffusion. If it were possible to assume in a given system that the chemical concentration, although spatially nonuniform, did not vary with time, then by the observation of the variation of the pattern of radioactivity and the application of these principles, which Dr. Householder and I chose to call interfusion rather than diffusion, something could be determined about the rates of exchange as they varied in space.

The trouble with this is that, like so many of the more sophisticated tracer experiments we have been doing lately, it is going to take a great deal of labor. In the first place the characterization of a spatially nonuniform chemical composition is going to be difficult. Perhaps histochemical methods could be applied to this. On the other hand, the radioactivity movements perhaps can be refined and methods can be worked out, as Drs. Dent and Hunt have shown here in their autoradiographic studies, whereby the movement of the tracer might be determined with sufficient precision.

WILLIER: I should like to emphasize one point which was more or less implied in Dr. Dent's remarks about the use of radioactive iodine in connection with the studies on the thyroid. The evidence indicates very clearly that the thyroid primordium, before it begins a transformation into follicles with storage of thyroglobulin, is capable of selectively accumulating large quantities of radioactive iodine. This indicates to me that cells of the primordium are already specialized in the direction of thyroglobulin-secreting cells. By means of this technique there is the possibility of tracing the initial time when these cells become specialized in a particular direction, i.e., with a specific selective affinity for iodine.

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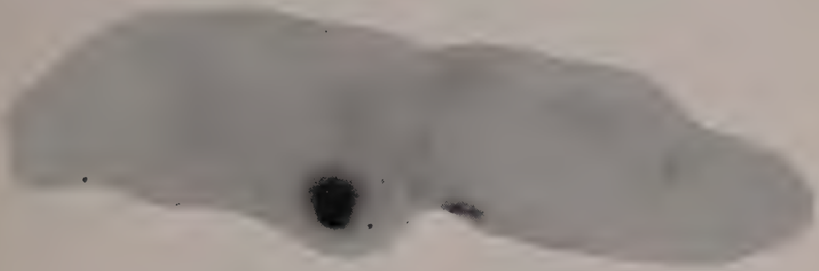
PLATES

PLATE 1

EXPLANATION OF FIGURES

1 Autoradiogram made from a parasagittal section of a tadpole *(Rana sylvatica)* which had been immersed in a solution containing P^{32}. A relatively dense concentration of P^{32} is demonstrated in a hind-limb bud (from unpublished work of Gennaro and Dent).

2 and 3 Whole mounts of 28- and 84-hour chicken embryos removed from eggs injected with P^{32} in solution were used to make the autoradiograms shown in these two figures (from unpublished work of Hunt).



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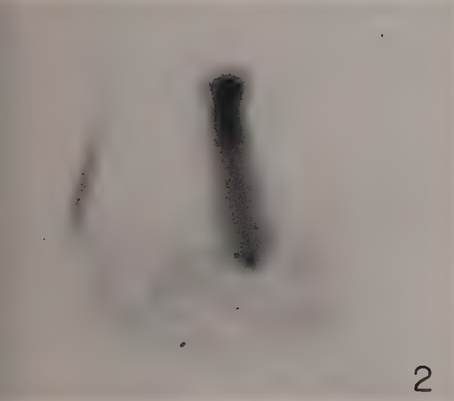

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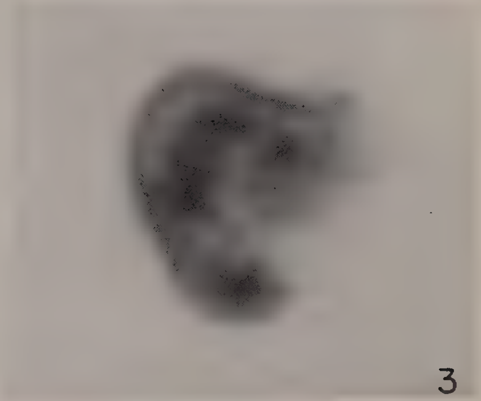

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PLATE 2

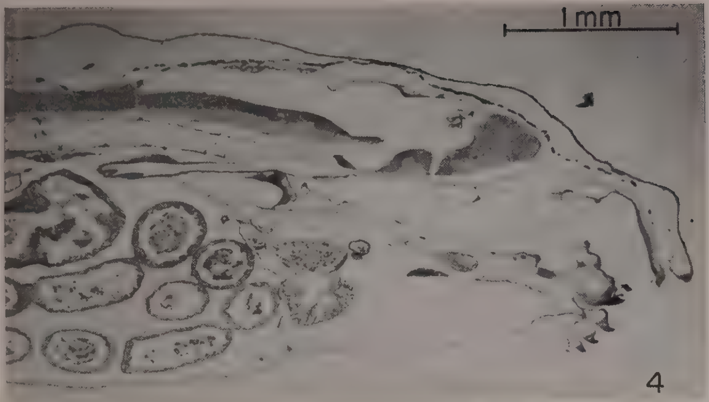
EXPLANATION OF FIGURES

4 Sagittal section through head and anterior trunk region of young tree frog tadpole. Tadpole has been immersed in a solution of radioiodine.

5 Autoradiogram prepared from section shown in figure 4. Note heavy localizations of radioiodine in horny teeth and beak at right and in thyroid in the center of the picture. The gut sections also show heavy localization. Weaker impressions of the heart, subdermal melanophores, and skin are evident.

6 Transverse section through the eye of another tree frog tadpole.

7 Autoradiogram of eye section depicted in figure 6. Heavy localization of radioiodine is apparent in the pigmented layer (figs. 4–7 from Dent and Hunt, '52).



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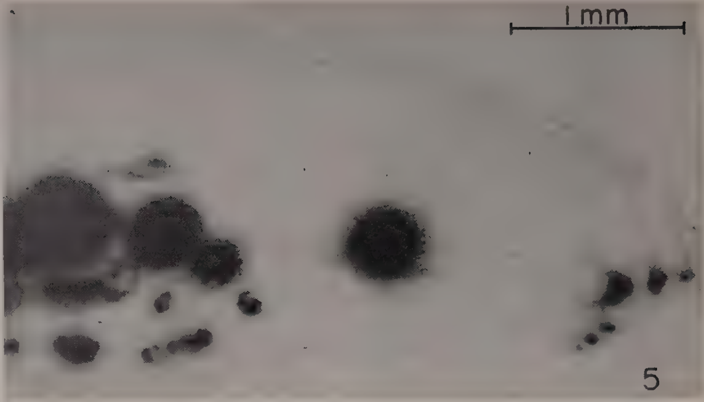

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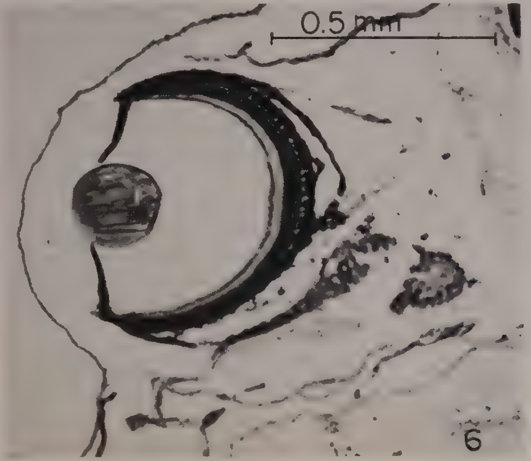

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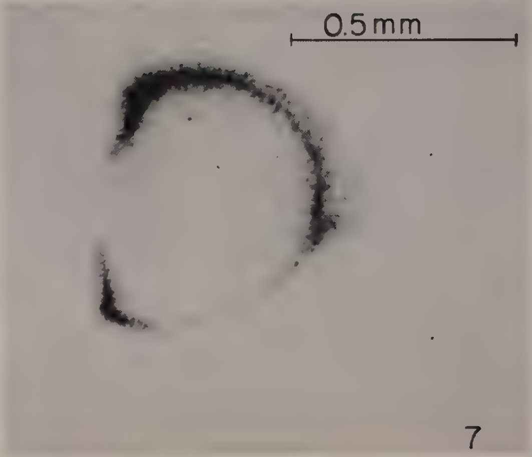

7

AN ANALYSIS OF THE CHANGING RADIATION RESPONSE OF THE DEVELOPING MOUSE EMBRYO[1]

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TWELVE FIGURES

INTRODUCTION

Our investigations with radiation as a deleterious agent in mammalian development have had three main purposes: (1) the study of the effects of radiation on developing systems as part of the general problem of radiation effects on living systems; (2) the use of radiation as a tool in experimental mammalian embryology; and (3) the application of experimental results to the practical problems encountered when human beings are irradiated. In this paper, our results will be presented from the first two points of view. The last has been discussed elsewhere (Russell and Russell, '52).

For investigating the effects of radiation on development (the first listed purpose, above), the mammal is not as suitable as is oviparous material, such as amphibia and invertebrates. The maternal environment adds difficulties, both to the observation of the embryo and to the interpretation of mechanism of radiation effect. However, as will be shown, these difficulties can be partially overcome.

For experimental mammalian embryology, the second listed purpose of our investigations, radiation is an excellent tool. The most obvious advantage over the use of injected materials is the fact that the "placental barrier" may be ignored, thus facilitating (a) accurate timing of the disturbances to the embryo itself; (b) accurate calculation of doses actually reaching the embryo. For the type of questions we have asked, perhaps the most important advantage of radiation lies in the fact that its action has a general distribution throughout the organism, so that selective response of structures may be expected to indicate patterns of sensitivity intrinsic to the embryo.

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[1] Work performed under Contract W-7405-eng-26 for the Atomic Energy Commission.

[2] Throughout most of the work reported, we have had the very able technical assistance of Mrs. Mary H. Major.

The literature has been reviewed extensively in another paper (Russell, '54). Most of the early investigations were made for use in human applications and lacked an important prerequisite that would have yielded answers to the more basic questions. This prerequisite is, of course, knowledge of the stage in development at which radiation is applied. At the time when our own investigations were begun, only a few papers were available in which accurate timing had entered into the design of the experiment (Kosaka, '27, '28a, b, c, d, e; Job *et al.*, '35; Kaven, '38a, b; Warkany and Schraffenberger, '47; Wilson, '49) and most of these were either restricted with respect to stages treated, or incomplete in the description of the types and incidences of abnormalities observed. As a contribution to the emerging pattern in what was, until recently, a rather diffuse collection of data in this field, this paper will present an analytical summary of a large portion of our work of the past 5 years, together with some speculation on mechanisms of effect.

METHOD

The basic method has been similar in most of our experiments. Total-body irradiation was administered to pregnant (or presumed pregnant) females at stages differing by 24-hour intervals and starting with day ½ after fertilization. Each animal was irradiated only once. Radiation factors were as follows: 250 kvp X rays with a half value of 0.4 mm of copper were delivered at approximately 80 r per minute as measured on the scatter block on which the animals were exposed; doses in different experiments ranged from 25 to 400 r.

For most of our experiments, the embryos used were F_1 hybrids between two inbred strains (C57BL mothers and NB fathers). This material combines genetic uniformity with hybrid vigor. To study the effect of genetic background, two other inbred strains, BalbC and 129, have also been used. All of the experimental embryos came from second litters. These are superior to first litters in various practical respects, such as littersize and ease of parturition.

Except in the preimplantation experiment, females were, in almost all cases, permitted to bring litters to term. Newborns were killed within 24 hours. They were weighed, measured, studied externally, dissected and processed for skeletal examination by a modification of Dawson's Alizarin-S technique.

In our main survey experiment the external features and skeletons of 420 experimental and 372 control young were studied in detail. For the combination treatments (radiation + hypoxia or chemical) we have obtained an additional 541. To study the action of radiation on quantitative characters within strains and on different genetic backgrounds, several thousand skeletons have been processed but as yet only partially examined. To elucidate radiation effects on preimplantation stages 831 embryos and resorbing bodies were observed.

EXPERIMENTAL FINDINGS

Upon plotting the main features of the results obtained with 200 r for all the stages studied (fig. 1), it becomes apparent that the gestation period may be conveniently divided into three broad phases. Irradiation during the first phase, which is the preimplantation period of the mouse embryo, causes a high incidence of prenatal mortality but virtually no abnormalities in the survivors. As susceptibility to prenatal death decreases, we enter the second phase during which sensitivity to neonatal death and sensitivity to gross abnormalities at term both come to their peaks. This second phase can be said to correspond to the period of major organogenesis of the mouse embryo. Finally, during the period of the fetus, sensitivity as measured by all three criteria drops to low levels. Since little more will be said in this paper about the period of the fetus (concerning which we have done only exploratory work), it may be noted here that, given different criteria — namely, histological changes or changes of function (e.g., fertility), rather than gross changes, and observation in later life rather than at birth — sensitivity can, of course, be demonstrated to be at levels higher than those shown here (Bagg, '22; Parkes, '27; Kosaka, '28b, c, e; Kaven, '38a; Hicks, '50, '53). In this paper, our results from irradiating preimplantation stages and stages during the period of major organogenesis will be summarized.

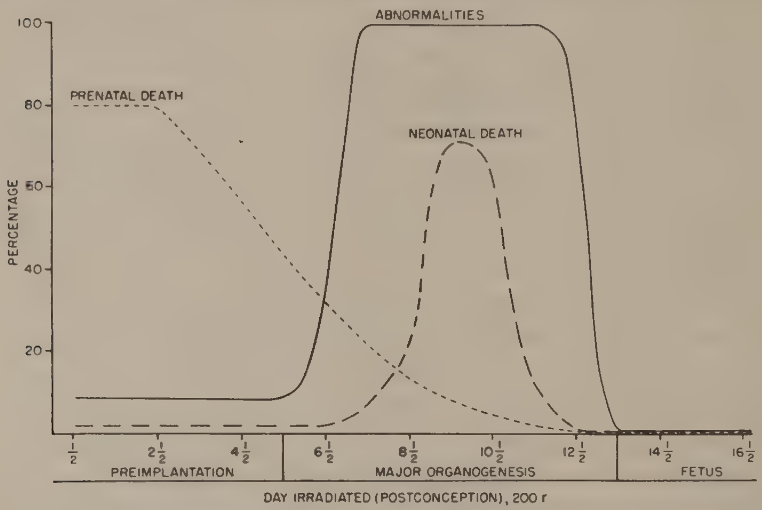

Fig. 1 Incidence of death and of abnormalities at term following irradiation with 200 r at various stages (separated by 24-hour intervals) in the prenatal development of (C57 × NB)F_1 mice. The incidence of abnormalities represented is calculated as percentage of animals possessing one or more. (In many cases, a single animal may have dozens of abnormalities; a few of the more detailed results appear in fig. 4.)

I. Mortality

A. Prenatal. Prenatal mortality may be manifested in the termination of pregnancies or in the death of individuals within surviving pregnancies. The former effect was measured by decrease in the proportion of copulations resulting in observable pregnancies (table 1), the latter by reduction in average size of litters coming to term (table 2).

TABLE 1

Percentage of copulations resulting in pregnancy in controls and following irradiation with different doses at different stages in the prenatal development of the mouse

DOSE (r)	OBSERVATION	DAY (POSTCONCEPTION) TREATED					
		½ – 4½		5½ – 8½		9½ – 13½	
		Pregnancy not diagnosable[c]				Diagnosed as pregnant[d]	
		No. ♀♀ treated	% w. litter	No. ♀♀ treated	% w. litter	No. ♀♀ treated	% w. litter
0	Term[a]	18	72.2	18	72.2	11	90.9
100	Term[a]	15	46.7	1	100	6	83.3
200	Term[a]	20	45.0	29	69.0	24	87.5
300	Term[a]	..	...	9	0.0	27	96.3
400	Term[a]	..	...	..	..	4	100.0
0	10½,13½ PC[b]	77	79.2	..	..	..	..
200	10½,13½ PC[b]	77	55.8	..	..	..	..

[a] Russell, '50; Russell *et al.*, '51.

[b] Russell and Russell, '50b. PC = postconception.

[c] At time of treatment.

[d] The fact that the percentage of treated females having litters at term is not 100 is probably due to occasional faulty diagnosis of pregnancy at time of treatment, although it is, of course, possible that there may have been some abortion or resorption.

Table 1 pools the results of three experimental series, all using (C57BL × NB)F$_1$ embryos. The data clearly indicate prenatal loss of litters as a result of 200 r delivered during the preimplantation period (days ½–4½) and possibly even following only 100 r (difference from control, however, not statistically significant for the small sample). On the other hand, for irradiation after implantation, there is no significant difference from the control with doses up to 200 r administered during the early part of the period of major organogenesis (days 5½–8½) and with doses up to 400 r administered during the later part (days 9½–13½).

Table 2 includes littersize data obtained as an incidental result in 6 different experimental series using different genetic strains. The pooling of series was done for the purpose of swelling the numbers in the stage-dose subgroups (total for table: 419 litters, 2667 young). The control littersizes for the different strains are quite similar, but, because of possible genetic differences in radiation response, the table should be interpreted with caution. Although the heterogeneity of the data does not permit simple statistical treatment, the following conclusions seem justified. Littersize at term is reduced as a result of irradiation during preimplantation stages (days ½–4½) both with 200 r and with 100 r. Irradiation shortly after implantation and early in the period of major organogenesis (days 5½–8½) affects littersize at term only if doses of 200 r and above are used, and still later irradiation (days 9½–15½) is without effect, except possibly with a dose of 400 r.

TABLE 2

Average size of litters coming to term following irradiation with different doses at different stages in the prenatal development of the mouse [a]

DOSE (r)			DAY IRRADIATED (POSTCONCEPTION)					
			½ – 4½		5½ – 8½		9½ – 15½	
	No. of litters	Litter size	No. of litters	Litter size	No. of litters	Litter size	No. of litters	Litter size
0	122	6.7 ± 0.18						
25			..	..	18	6.4	..	..
50			..	...	23	6.0	..	..
75			..	..	7	7.0	..	..
100			7	5.1	46	6.7	46	7.2
200			11	4.3	36	5.1	41	6.7
300			2	4.5	3	1.0	51	6.5
400			..	..	..	..	6	5.2

[a] Pooled data from Russell, '50; Russell and Russell, '50a; Russell *et al.*, '51.

Thus, the birth data indicate that irradiation of postimplantation stages causes considerably less prenatal death than does preimplantation treatment; that, during the period of major organogenesis, sensitivity to prenatally lethal effects decreases rapidly; and that what lethal effects there are for that period are primarily due to death of individuals rather than termination of entire pregnancies. The birth data for preimplantation irradiation, on the other hand, point to considerable incidence of both types of loss. For that reason, a separate experiment involving examination of the uterine contents either 10½ or 13½ days after copulation was carried out for these stages with 200 r of X rays. Each irradiated presumed pregnant female was paired with a strictly comparable control handled simultaneously (Russell and Russell, '50b).

Prenatal death induced by 200 r was found to be considerable. The average number of living embryos per irradiated female was only about 20% of the controls in groups irradiated on day ½, 1½, or 2½; following treatment on days 3½ and 4½, it was 31 and 57% respectively (fig. 2 A). (This should be compared with 76% survival for days 5½–8½ and probably 100% survival for later stages — tables 1 and 2.) All deaths occurred before day 10½, mortality between days 10½ and 13½ and day 13½ and term being no higher than in controls. In agreement with the much scantier data from observations made at birth, the dissection results show that prenatal mortality resulting from irradiation of preimplantation stages may be manifested either in (1) termination of pregnancies or in (2) the death of individuals within surviving pregnancies.

1. Early termination of pregnancies, as indicated by the highly significant excess of nonpregnant females in the irradiated groups at the time of dissection (fig. 2 A), accounts for the loss of almost 30% of the irradiated embryos (see lower half of table 1). This whole-litter loss could be due to (a) direct radiation death of every embryo in a uterus;

or (b) direct radiation death of a number of embryos sufficiently large to exert an adverse effect on the viability of the rest of the embryos in the uterus; or (c) radiation effect on the mother which prevents continuation of a pregnancy. When the distribution of uteri with regard to the number of implants is plotted for any one stage, the "zero" category is found to be excessively large as compared with categories of 1, 2, 3, etc., indicating that hypothesis (a) cannot account for all the effect. The fact that, as can be seen from figure 2 A, there is no obvious correlation between the averages for total death and death due to loss of whole litters for irradiation on different days indicates that hypothe-

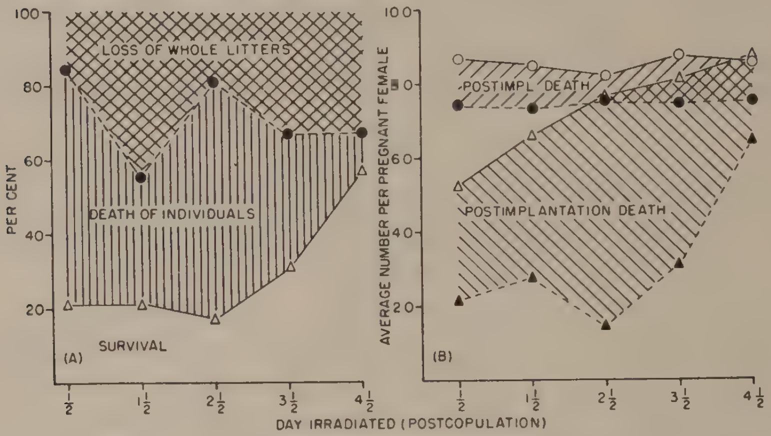

Fig. 2 Effects of irradiation with 200 r during the preimplantation stages of the mouse embryo. All observations are made on day 10½ or 13½ postcopulation, and points are based on a total of 521 implants in the controls, 310 in the irradiated groups (Russell and Russell, '50b; Russell, '54).

(A) △———△ yield of living embryos per treated female, expressed as percentage of controls. ●-----● proportion of matings resulting in any implants, expressed as percentage of control proportion.

(B) Based on pregnant females only. ○———○ implants per control; △———△ implants per irradiated female; ●-----● living embryos per control; ▲-----▲ living embryos per irradiated female. The space between the solid lines represents preimplantation death of individuals in surviving pregnancies. (By permission from *Radiation Biology*, Vol. I, edited by Alexander Hollaender. Copyright, 1954. McGraw-Hill Book Co., Inc.)

sis (b) is unimportant. It therefore appears likely that hypothesis (c) accounts for some of the loss of whole litters.

2. Death of individuals within surviving pregnancies accounts for about two-thirds of the total death in groups irradiated day ½, 1½, or 2½ but becomes a much smaller portion of the total loss when over-all mortality decreases (fig. 2 A). As is shown in figure 2 B, based on pregnant females only, individual embryos may die before implantation if irradiated shortly after conception, accounting for the loss of 3.3 and 1.9 implants per pregnancy surviving irradiation on days ½ and 1½, respectively. On the other hand, death of individuals after implantation is considerable for all of the preimplantation stages irradiated. This postimplantation death occurs quite early since by day 10½ the features of dead embryos are no longer recognizable. Therefore, until observations are extended to stages earlier than day 10½, we shall be in ignorance as to what morphological abnormalities, if any, are produced in embryos killed by preimplantation treatment. The survivors, as already stated, are virtually normal.

In summary, irradiation during the preimplantation period causes a high incidence of prenatal loss, due to both termination of pregnancies (probably largely an effect on the mother) and death of individuals within surviving pregnancies. For comparable doses, irradiation during the period of major organogenesis causes considerably less prenatal death than does treatment during the preimplantation period. *Within* the period of major organogenesis, susceptibility to prenatal killing probably decreases fairly rapidly with embryonic age.

B. Neonatal. The contrast between pre- and postimplantation irradiation is in the opposite direction with regard to neonatal death (stillbirth or death within 24 hours). The neonatal death is not in excess of controls in animals born following 200 r treatment given between days ½ to 6½ inclusive (fig. 3). Neonatal mortality is at a peak following irradiation on days 9½ and 10½. The 300 r data in general parallel the 200 r data in this respect and reach 100% for the two most susceptible stages. Doses of 100 r and below

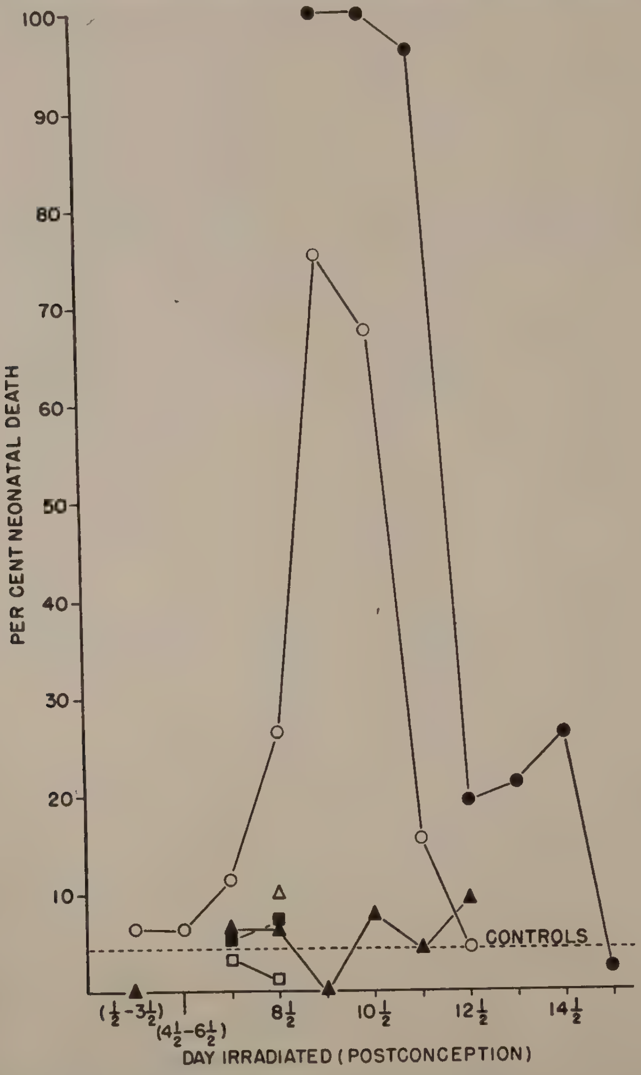

Fig. 3 Incidence of neonatal death following irradiation with different doses at different stages in the prenatal development of the mouse. ● ——— ● = 300 r; ○ ——— ○ = 200 r; ▲ ——— ▲ = 100 r; △ = 75 r; ■ ——— ■ = 50 r; □ ——— □ = 25 r (from Russell, '54).

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have apparently no effect on neonatal death. It is, therefore, possible to set the neonatal LD_{50} for irradiation on:

days $\frac{1}{2}$–$8\frac{1}{2}$ at > 200 r,
days $9\frac{1}{2}$, $10\frac{1}{2}$ at between 100 and 200 r,
day $11\frac{1}{2}$ at between 200 and 300 r,
days $12\frac{1}{2}$–$15\frac{1}{2}$ at > 300 r.

All mortality data presented may be roughly summarized in table 3.

TABLE 3

Summary of the effects on viability of prenatal irradiation of the mouse

	DAY IRRADIATED (POSTCONCEPTION)			
	$\frac{1}{2}$ – $4\frac{1}{2}$	$5\frac{1}{2}$ – $8\frac{1}{2}$	$9\frac{1}{2}$ – $10\frac{1}{2}$	$11\frac{1}{2}$ – $13\frac{1}{2}$
Effect on prenatal viability:				
(a) Termination of pregnancy	+++	+	0	0
(b) Death of individuals	++++	++	+	0
Effect on neonatal viability	0	++	++++	+

II. Sex ratio

There is one claim in the literature (Job *et al.*, '35; discussed in greater detail by Russell, '54) that irradiation between the ninth and eleventh days in the rat produces an excess of males, namely, 62%. No control figures are cited. We are unable to support this for any of our groups irradiated postimplantation or for the total of all such groups (Russell, '50). It may be pointed out here that the percentage of males in one of our *control* groups was 61%.

An indication of possibly (though still not significantly) upset sex ratio that we had from the irradiation of preimplantation stages was not supported by a repetition of the experiment (Russell, '50).

Although it is, of course, quite conceivable that the sex ratio at birth might be affected by prenatal irradiation — either through differential mortality of one sex or through actual sex reversal — valid reports of such effects must to date be considered lacking. To detect even relatively great effects on sex ratio, it is necessary that experiments be done on a very large scale and with completely comparable controls.

III. Birth weight

In a population of genetically homogeneous litters of uniform parity, it was found that mean birth weights for animals irradiated with 200 r on days 8½ through 13½ postconception were significantly lower than control birth weights (Russell, '50). The 300-r curve was, in general, parallel to the 200-r curve with a mean difference of 0.2 g between the two. Minima for both curves lay between the 10½- and 11½-day stages. The short 400-r curve available, paralleled the 300-r curve in the rising portion between days 12½ and 13½.

Holding the time of irradiation constant, a more complete dose-weight series was recently obtained for the stage most susceptible to general growth retardation, i.e., day 11½ (fig. 6 A). Weight reduction per 100 r averages 0.22 g over the three available intervals.

IV. Morphology

Even within the relatively limited literature on the subject, a large number of abnormalities has been reported as resulting from the irradiation of mammalian embryos (see Russell, '54, for review). Unfortunately, only a few authors have recognized the importance of considering the stage of treatment. Some of these chose to concentrate on one or a few stages (Raynaud and Frilley, '43a, b, '47a, b, c, '49a, b; Wilson *et al.)* or specific types of abnormalities (Kaven, '38b). Others, namely, Kosaka, Job *et al.,* Kaven ('38a), Warkany and Schraffenberger, Hicks ('53), have, instead, surveyed considerable portions of the gestation period and their work shows the probable existence of critical periods.

A considerable portion of our work was designed to map carefully the changing sensitivities to the induction of a large number of abnormalities in order to draw some conclusions about critical periods in general as well as to provide specific information that would be useful to embryologists and developmental geneticists. It may be repeated here that the main emphasis was placed on the skeleton although the external features were also studied and limited information was obtained on the eyes and the urogenital system.

It is obviously impossible to describe in detail the well over one hundred types of abnormality studied. This section will, therefore, be limited to a discussion of the general features of the results, illustrated by a selected few of the abnormalities for specific points of interest.

Figure 4 shows a sample of the yield of skeletal abnormalities resulting from the irradiation of embryos. Elsewhere we have illustrated several other skeletal results (Russell, '54) and have described external and visceral abnormalities (Russell, '50). No detailed morphological descriptions will here be given, but may be found in other publications (Russell, '49, '55).

The abnormalities illustrated in figure 4 were observed at birth. In the diagram, however, the results are projected back onto the stage at which irradiation was applied. Each band, whose width represents percentage incidence of abnormality (see scale in diagram), thus indicates the time distribution throughout embryonic development of sensitivity to a particular change. Absence of a solid band anywhere between days ½ and 12½ indicates lack of response to a dose of 200 r, or below.

A. General features of the results. As has already been mentioned, there is a virtual absence of abnormalities in the survivors from 200 r irradiation given at very early stages (days ½–5½). This holds true for all the characters investigated, including many not shown in figure 4. At the other end of the time scale, on day 13½, even 300 r produces few abnormalities (Russell, '49, '50) and it is probable that 200 r would not cause any obvious gross changes. In the intermediate range, days 6½–12½ inclusive, 200 r is amply sufficient to produce a high incidence of malformations (see section V for changes produced by lower doses). With this dose, the percentage of animals with one or more gross abnormalities is 100 for each of days 7½–11½.

It is apparent from figure 4 and other illustrations of a similar type (Russell, '50, '54) that when the lowest dose that gives an easily detectable incidence of a particular abnormality is used, the bulk of that incidence usually occurs as a result of irradiation on 1 or, more rarely, 2 days. The time interval during which there is sensitivity to the induction of a particular abnormality as observed at birth, will be referred to as the "critical period" for that abnormality. At any one stage, during major organogenesis, a number of different critical periods come to their peak (fig. 4; Russell, '50, fig. 7), and no single stage stands out unequivocally as the most sensitive one for the embryo as a whole.

The fact that the critical period extends over stages just preceding or following that of maximum sensitivity may be explained by either or both of two hypotheses: (a) wide deviation of a few individuals from the average developmental age of a population of given chronological age; (b) real increase and decrease in sensitivity of certain specific processes. The latter is supported by several instances of chronological extension of sensitivity on use of higher doses (see arch-centrum approach, foot reductions, etc.). This extension may be in both directions, although the present data provide fewer examples of gradual increase in sensitivity because, on account of excessive mortality, the 300-r series was not carried to stages earlier than day 9½. It is important to bear the high-dose extension effect in mind when making comparisons with other teratogenic agents applied

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Fig. 4 Critical periods for the induction of a few of the well over 100 types of abnormality observed at term following prenatal irradiation. Representation is by (a) percentage incidence of abnormality (see scale in figure), and (b) magnitude of dose required to produce abnormality. Thus, the wider and more heavily shaded a band, the greater the sensitivity. Absence of a band at a particular stage indicates that the abnormality did not occur in the irradiated group, except where the serrated end of a band indicates that the dose series was not continued to the stage in question (see also bottom of figure showing extent of different dose series). (Data from Russell, '49, '50, '54.)

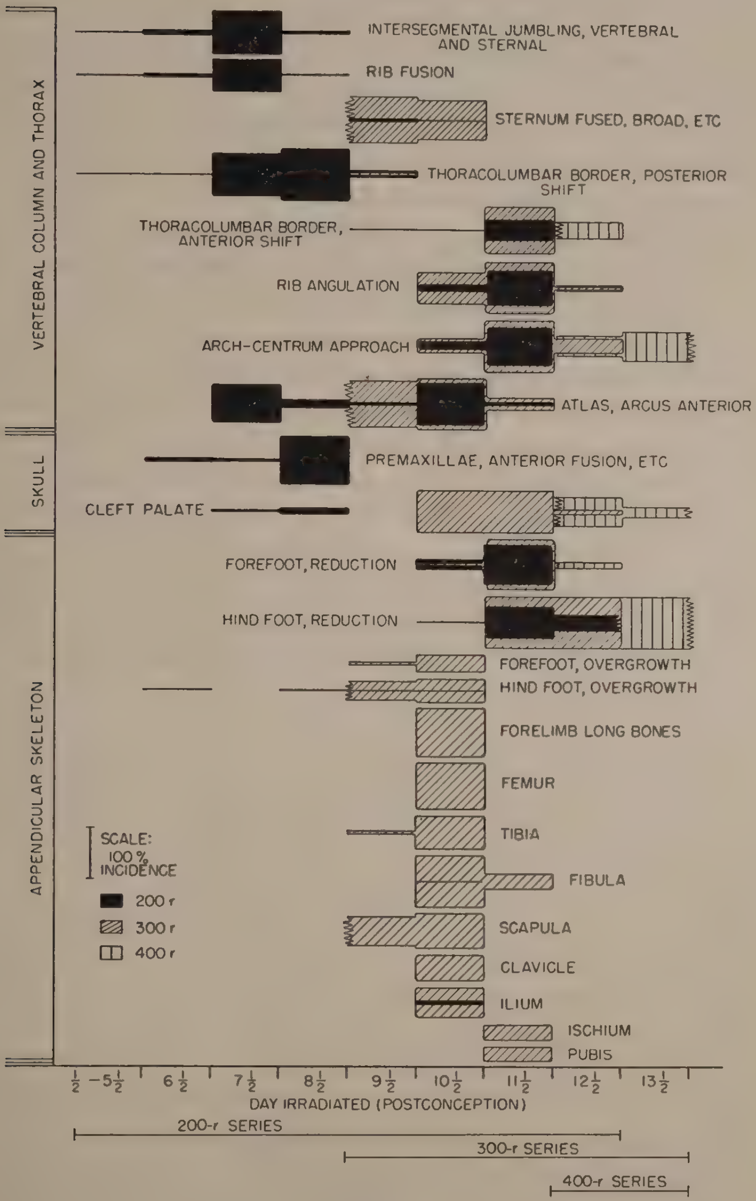
VERTEBRAL COLUMN AND THORAX
INTERSEGMENTAL JUMBLING, VERTEBRAL AND STERNAL
RIB FUSION
STERNUM FUSED, BROAD, ETC
THORACOLUMBAR BORDER, POSTERIOR SHIFT
THORACOLUMBAR BORDER, ANTERIOR SHIFT
RIB ANGULATION
ARCH-CENTRUM APPROACH
ATLAS, ARCUS ANTERIOR
SKULL
PREMAXILLAE, ANTERIOR FUSION, ETC
CLEFT PALATE
APPENDICULAR SKELETON
FOREFOOT, REDUCTION
HIND FOOT, REDUCTION
FOREFOOT, OVERGROWTH
HIND FOOT, OVERGROWTH
FORELIMB LONG BONES
FEMUR
TIBIA
FIBULA
SCAPULA
CLAVICLE
ILIUM
ISCHIUM
PUBIS
SCALE: 100 % INCIDENCE
200 r
300 r
400 r
½ −5½
6½
7½
8½
9½
10½
11½
12½
13½
DAY IRRADIATED (POSTCONCEPTION)
200-r SERIES
300-r SERIES
400-r SERIES

Figure 4

at the same stage since merely quantitative changes in radiation may lead to qualitatively different results. Thus, while low-dose irradiation at a certain stage produces changes only in characters at the peak of their sensitivity, treatment with higher doses calls forth additional abnormalities, with peak sensitivity at other stages, which may modify the expression of the first set of changes.

Apart from the extension effect, higher dose results indicate the same locations of sensitive stages as do those for lower doses, with incidence of abnormality changing in an approximately parallel manner from stage to stage. Increase in dose almost always leads to increase in incidence (fig. 4) and degree. Explanations for a few possible exceptions have been discussed (Russell, '50). The dose-incidence slope at the stage of maximum sensitivity is different for different abnormalities. For example (see fig. 4), the 200 r and 300 r incidences for anterior shift at the thoracolumbar border are 40 and 88%, respectively; for abnormalities of long bones of the forelimb, they are 0 and 93%, respectively. (Note: The numerous cases in which 100% incidence has already been reached at 300 r are obviously not suitable for comparisons of this type.)

Certain abnormalities have two critical periods. Clear examples are cleft palate (fig. 4), changes in the arcus anterior of the atlas (fig. 4), and shortening of the tail (fig. 7, Russell, '50). In some cases the abnormalities produced during the two phases may be somewhat different (e.g., arcus anterior); in others (e.g., cleft palate), they are themselves similar (at least at term — there remains, of course, the possibility of differences apparent at later or earlier observation), although they differ in correlated changes. The occasional similarity of end result is interesting in view of the marked difference in developmental states at the times of the initial disturbance. It is possible that the few instances of apparently long critical periods (e.g., tail malformations — Russell, '50; nonossification of cervical centra — Russell, '55) are actually compounded of two or more phases not as sharply separated as they are in the above examples.

B. Comparisons with the effects of other agents. Turning to specific radiation-induced abnormalities, a few will be singled out for comparison with the effects of other agents altering embryonic development, namely: (1) certain mutant genes; (2) "intangible" environmental factors; (3) certain chemical teratogenic agents recently used in genetically controlled mouse material. In keeping with the summarizing nature of this paper, the discussion will be kept brief. Systematic treatment of anatomical effects with descriptive detail and illustrations may be found in other publications (Russell, '49, '50, '55).

1. There are several instances of approximate correspondence between the developmental effects of a mutant gene and the syndrome of changes produced by irradiation at a given stage. Irradiation on day 7½, for example, results in a complex set of changes in cervical and thoracic vertebrae and in the thorax termed "intersegmental jumbling" (fig. 4), which is apparently due to interference with orderly segmentation, the two lateral halves acting independently (fig. 9). A gene, *stub* (Dunn and Gluecksohn-Schoenheimer, '42), produces effects of a similar type, but the disturbance is severer as well as more extensive, for, instead of merely affecting the prelumbar region it causes jumbling over the entire vertebral column. On the other hand, *stub* animals do not suffer from abnormalities of the skull, the eyes, or the atlas, all of which are obtained, in addition to "jumbling," with considerable frequency as a result of irradiation on day 7½.

Irradiation on day 9½ elicits a set of symptoms (tail abnormalities, urogenital and anal defects, spina bifida) copying part of the action of a sizable group of "tail" genes with similar phenotypic effects (see Russell, '50, '55, for more detailed comparison). In addition, it produces a set of abnormalities roughly paralleling most of the manifestations of the *screwtail* gene (MacDowell *et al.*, '42). Among the latter group of defects are: short, broad, fused sternum;

ventrally reduced and often thinned ribs; tail deformities; defects in lumbar and thoracic centra; and certain abnormalities in mandible and cranium (fig. 10). Still other changes produced with high incidence by irradiation on day 9½ (e.g., various eye defects) are not included in the syndromes, either of *screwtail* or the group of other "tail" genes referred to above.

The foregoing examples illustrate certain features that emerge in most of the comparisons between gene and radiation effects, namely (i) that the processes that are disturbed by irradiation at a given stage are more numerous than those affected even by a highly pleiotropic mutant gene, e.g., *screwtail,* and (ii) that this more widespread action of radiation exists even for doses that produce a less severe change than the gene.

While there are several instances in which all of the abnormalities produced by a given mutant have critical periods (as demonstrated in the radiation experiment) coinciding at the same stage, there are at least two pleiotropic genes, *undulated* and *short-ear,* that cause syndromes whose component parts cannot be copied by irradiation at any one or even two stages (Russell, '55). The action of both of these genes has been ascribed to generalized disturbances, namely, reduction of the entire mesenchymatous preskeleton for *undulated* (Grüneberg, '50), and reduction in the rate of cartilage formation for *short-ear* (M. C. Green, '50, '51). It, therefore, appears unlikely that irradiation at one stage is capable of producing similar damage to whole tissues of these types. The selective action of radiation probably has a different basis (see our Discussion).

2. Differences in certain characters that show variability *within* inbred strains are determined by environmental factors acting during embryonic development. In the case of homeotic changes in the axial formula of the mouse, these factors have been analyzed (E. L. Green, '41, '51) and found to be largely "intangible" (i.e., only a small portion of the variability can be resolved into such tangible components as

age of mother, season of year, etc.). Radiation is evidently capable of copying the action of these intangible environmental factors since prenatal treatment can produce shifts both at the thoracolumbar (fig. 4) and the lumbosacral borders (Russell and Russell, '50a). It is of interest that our method has revealed that two processes may govern the formation of what has heretofore been considered — in mathematical treatments of quantitative inheritance — a singly determined character: the thoracolumbar border is apparently sensitive to posterior shift on days 7½ to 8½ and to anterior shift on day 11½ (fig. 4).

3. Of the number of injected teratogens tested in mammals, two, recently used on genetically controlled mouse material, may be chosen for the most direct comparison with radiation data. Trypan blue, injected into pregnant females 7–8 days after fertilization (Hamburgh, '52; Waddington and Carter, '52), produces failure of anterior neural tube closure and death before day 12½, as well as tail abnormalities visible in survivors at term. The former of these abnormalities, if studied in detail, may prove to be similar to Kaven's "extrakranielle Dysencephalie," produced by irradiation of the same stages (Kaven, '38b). On the other hand, the earliest stage at which tail abnormalities can be produced by irradiation is day 8½ (Russell, '50) or day 9 (Kaven, '38a), and incidence for such early treatment is comparatively low. This may indicate either a real difference between the teratogens or a persistence of the trypan blue action beyond the moment of injection until the stage indicated to be sensitive by the radiation data. The failure of trypan blue to elicit many abnormalities brought about by irradiation on day 7½ (or even at later stages if there actually is persistence) has been pointed out (Waddington and Carter). Part of this difference may be merely a matter of observation, since the major 7½-day radiation abnormalities are not externally apparent and skeletal examinations of the trypan blue-treated animals were not carried out. On the other hand, it is quite possible that a poison, such as trypan blue, has affinities for

certain parts of the embryo so that its very earliest action is not as randomly distributed as are the ionizations produced by X rays.

Malformations produced by cortisone (Fraser and Fainstat, '51) and 17-hydroxycorticosterone (Kalter and Fraser, '52) are apparently also of more limited variety than those called forth by X rays at comparable stages of development. For doses of cortisone which gave a high incidence of cleft palate, the main abnormality studied, only three other malformations were listed, even though the effects of the compound were tested over a range of stages. Noteworthy is the absence of foot and tail abnormalities which are produced by X rays with considerably greater incidence than is cleft palate for treatment on day 11½ (fig. 4; Russell, '50, fig. 7), a stage for which the incidence of cortisone-induced cleft palate is very high. It is of interest that the type of palate abnormality is the same for both teratogens (the cleft occurring always in the midline and involving regions only posterior to the premaxillae), while it differs from two types of genetic cleft palate described in the mouse (Reed and Snell, '31–'32; Gluecksohn-Schoenheimer and Dunn, '48). Radiation results show days 10½ and 11½ to be most sensitive to the induction of uncomplicated cleft palate (cleft palate produced during the early critical period, days 7½ and 8½, is always associated with a high degree of premaxillary fusion; fig. 4); and with cortisone, too, by far the highest incidence of cleft palate occurs if the four daily treatments include days 10 and 11 (i.e., if treatment is started day 8, 9, 10, or 11). However, considerably later injections may also produce a few instances of the abnormality and in those cases Fraser and Fainstat have suggested a degenerative change rather than delay in a developmental process.

C. Embryological basis of sensitivity. One of the ultimate aims of our investigation is to correlate end result observed with changes occurring in precursor processes at the stage demonstrated to be sensitive for the induction of the particular abnormality. Much of this type of interpretation will have to await direct observation of processes taking place at the time of irradiation or shortly after. However, until we have such data, certain events, known from descriptive embryology (Snell, '41; Grüneberg, '43) to occur at certain critical stages, may be used for provisional interpretations. Such a procedure reveals that, in the case of some abnormalities, sensitivity may be linked with certain events in visible primordia, while, in other cases, the correlations are more complex, sensitivity involving either very early precursors or surprisingly late stages. Some examples follow.

There are some abnormalities which are apparently caused by damage to the primordia. Thus, the second phase of sensitivity to tail reduction corresponds to a time of rapid growth of the tail bud; the critical periods for digital reductions in fore- and hind feet occur at the time of beginning footplate differentiation in fore- and hind-limb buds, respectively. It is also to be noted that abnormalities of all the other parts of the appendicular skeleton (long bones and girdles), with the exception of ischium and pubis, are produced either exclusively or with highest incidence about a day before the stage yielding the highest incidence of digital reductions (fig. 4). Thus, in these cases, too, there are indications that sensitivity is linked with a high degree of activity in the primordia themselves.

For quite a number of abnormalities, sensitivity involves early precursors of the character finally studied. For example: in the case of intersegmental jumbling, the critical period is at the time of the very beginning of primitive streak activity; the first phase of sensitivity to tail reduction comes at a time preceding the formation of a tail bud; and overgrowth of the digits may be obtained by irradiating not only prior to the formation of limb buds but even, in the case of hind feet, prior to the establishment of the postlumbar somites of the embyro. On the other hand, sensitivity may occur surprisingly late. For example, a certain abnormality in which the vertebral column may end as far forward as the ilium (''absence of terminal axis,'' see Russell, '55) has a peak critical period on day 11½, a stage at which a sizable tail bud containing numerous somites has already been formed. Perhaps the best example of unpredictability of sensitivity is the position of the thoracolumbar border (Russell and Russell, '50a) for which there are two widely separated critical periods (fig. 4): one, for posterior shift, occurring during somite segmentation but prior to the time when the most obviously involved somite (No. 21) is being formed; the other, for anterior shift, probably correlated with early precartilage formation in the fully completed complement of body somites.

V. Effect of genetic background

Using three different populations, each genetically uniform in itself, we are studying the effect of genetic constitution on radiosensitivity to the induction of homeotic shifts in vertebral borders and related changes in the thorax. Detectable shifts can be obtained with doses as low as 25 r. The comparison between strains, which is being done on a large scale with 100 and 200 r, is still in progress, but some of the data already obtained for the thoracolumbar border are shown in figure 5.

It will be noted, first, that the three strains have different control distributions and, second, that, as has been mentioned earlier, the most sensitive stage for the induction of posterior shift (production of > 13 thoracic vertebrae) comes before the most sensitive stage for anterior shift (production of < 13). If attention is focused on the $(C57 \times NB)F_1$ and 129 strains, and if the results obtained in these two strains for irradiation on day 8½ are compared, it appears that the $(C57 \times NB)F_1$ is more sensitive to induction of posterior shift of the thoracolumbar border. In contrast with this, if the results for day 9½ are compared, it appears that the 129 strain is more sensitive to anterior shift. However, the variation within strains and the differences between strains in the controls indicate that the final meristic expression of the character, as number of vertebrae, results from developmental thresholds at particular levels in a continuous array of variation in underlying processes. Therefore, the apparent differences in sensitivity are not necessarily the result of differences in sensitivity to primary radiation damage.

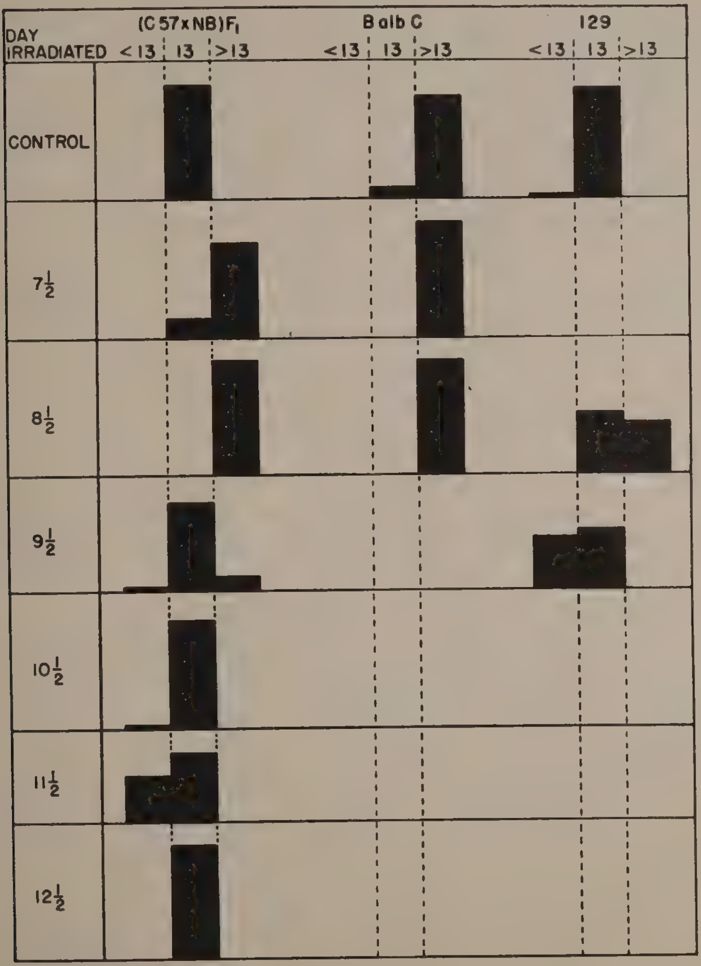

Fig. 5 Incidence of sides of animals having fewer than 13, 13, and more than 13 ribs in three strains of mice following irradiation at different stages in embryonic development. (Preliminary data, with several groups still to be filled in, representing only about one-fourth of 2012 newborns obtained for this experiment. Russell and Russell, '50a.)

The primary radiation effect could be of approximately the same degree in all three strains, and the final expression of this effect in any one strain could depend on the position of the strain on the scale of developmental potencies. Judging the positions of the strains on this scale by the frequencies marked off by the thresholds for < 13 and > 13 in the controls, the orders of effect in the three strains on the various days are, in fact, exactly what would be expected.

The results of this experiment have been given primarily to point out the need for caution in drawing conclusions about relative sensitivities, particularly with regard to characters that are alternate in final expression, but for which there is underlying continuous variation in developmental processes. Greater effect in the end result does not necessarily indicate greater primary radiation damage.

DISCUSSION

As was mentioned in the introduction, our results may be used both from the points of view of (1) shedding some light on the mechanism of radiation effects on developing systems and (2) shedding light on mammalian embryology with radiation used merely as a tool.

A result of considerable interest from the second point of view is the demonstration of great regulatory powers of early mammalian blastomeres. It may be recalled that irradiation during preimplantation stages causes a high incidence of early death but very few abnormalities in survivors. This finding, demonstrated in the mouse (Russell, '50; Russell and Russell, '50b), confirms indications of earlier experiments (see Russell, '54, for review) and has found corroboration in recent work on the rat (Wilson, Brent, and Jordan, '53; Hicks, '53). On the assumption, discussed later, that the initial effect of radiation is death of cells, let $p =$ probability of killing one blastomere or its immediate descendants by radiation. Minimum and maximum estimates of p, determined from limiting values for the factors considered in hypotheses (a), (b), and (c) (see pp. 109–110) may be obtained from the results of irradiating at the one-cell stage, i.e., on day ½. Assuming p to be equal in all early cleavage stages, it is possible to calculate, for a stage at which there are n blastomeres, the proportion of embryos with 1, 2, 3, . . . n cells killed. Taking either the minimum or maximum estimate for p, comparison of the arrays of calculated proportions with the observed incidence of mortality of embryos following irradiation at the corresponding time postconception indicates that the group surviving radiation includes embryos in which a considerable proportion of the blastomeres were killed. Since the survivors are normal, it may be concluded, if the above assumptions are correct, that the early mammalian embryo possesses great regulatory powers.

Embryological interpretations from the morphological data are best made for individual abnormalities in more detailed studies. The remainder of the discussion will, therefore, be devoted to speculations concerning our first listed problem, i.e., the mechanism of radiation effects on developing systems.

When considering the nature of the radiation effect it is important to examine the possible intermediary role of the maternal organism in any of the responses of the embryo. While such an intermediary role seems probable in the case of embryonic death manifested in prenatal loss of whole litters (see Results, section I.A), it is, on the other hand, probably legitimate to rule out effects on the mother as a factor in the production of most gross abnormalities. Evidence for this assumption comes from the similar effects obtained where the maternal intermediary is absent (e.g., in amphibian embryos) or practically circumvented through shielding (Wilson and Karr, '51). Circumstantial evidence for the unimportance of intermediate maternal effects in the genesis of most abnormalities is also supplied by the shortness of critical periods (discussed by Russell, '50) and by the rapidity of detectable response to radiation in embryonic tissues (Hicks, '50).

Assuming, then, that at least a large part of the damage is due to the action of radiation on the embryo itself, two major features of the results must be explained. These are: (a) although radiation is generally distributed through the embryo it causes selective damage which is consistently dependent on the stage irradiated, and (b) the selected parts respond in a consistent manner, at least within a narrow range.

Before advancing any hypotheses of basic radiation action, it should be made quite clear that use of the term ''critical periods'' does not imply that the immediate primordium of the character malformed as a result of irradiation at a certain stage was damaged at that stage, i.e., during the critical period. There are many cases of abnormalities that are obviously secondary to other abnormalities (e.g., sternal malformations, which are probably due to faulty growth of ribs, which, in turn, results from vertebral defects). There are probably even more cases in which the final malformations are the results of more obscure and as yet unsuspected chains of reactions. What a ''critical period'' does indicate is that the initial disturbance that touches off a chain of processes, regardless of their complexity, must occur at a certain stage to give a certain end result. In addition to localizing chronologically the beginning of teratogenesis, the specificity of end result revealed by the ''critical periods'' indicates differential sensitivity at some step (to be discussed later) in the chains of processes which proceed between the moments of irradiation and observation. It may be suggested that, in the case of diphasic critical periods (see Results, IV.A) the genesis of the abnormality induced during the first phase is a very indirect one, while irradiation during the second phase causes damage to the primordium itself.

What the nature of the initial action of radiation on the embryo is, remains a question on which there is at present no direct information. From the results of varied work on other organisms it may be concluded that the biologically effective damage of the generally distributed ionizations is probably in the nucleus of cells since doses of considerably greater magnitude than those employed in this experiment are required to cause detectable cytoplasmic changes. Among possible nuclear responses, somatic *gene* mutations (suggested by Wilson and Karr, '51; Wilson, Jordan, and Brent, '53) may be ruled out, because, in order to account for consistency of response, it would be necessary to assume either directed mutations or nonspecific cell lethals. The former assumption is contradictory to the findings of radiation genetics; the latter may be rejected because the frequency of *gene* mutations with dominant cell-lethal effects is probably very far below that needed to account for the observed incidence of embryonic abnormalities (see Russell, '54, for discussion). The most plausible interpretation is that the primary intracellular effect of radiation on the embryo is the induction of somatic chromosomal aberrations which result either in a breakdown of the mitotic process or in aneuploidy after cell division, the end result in both cases being a cell-lethal action. This explanation reconciles the observed consistency of response with the randomness of intracellular damage which must be postulated. In the above suggested chain of events, ionizations → chromosome breaks → chromosome aberrations → failure at mitosis or aneuploidy after mitosis → death of cells, the general phrase "primary intracellular damage" used in this discussion would refer to chromosome breakage, and the phrase "initial cellular effect, or damage" to death of cells.

In connection with this hypothesis, it is of interest that hypoxia protects markedly against radiation-induced abnormalities (fig. 6), the magnitude of protection being similar for several characters studied so far (Russell *et al.*, '51). The protective action of hypoxia against the radiation induction of chromosome aberrations is well known. However, since hypoxia also decreases the magnitude of other types of radiation damage, e.g., mitotic inhibition, this experiment does not yield conclusive proof concerning the nature of the primary damage to the embryo.

Again it should be made clear that the hypothesis of cellular death through chromosome aberrations is applied only to the basic radiation damage. The types of cellular change

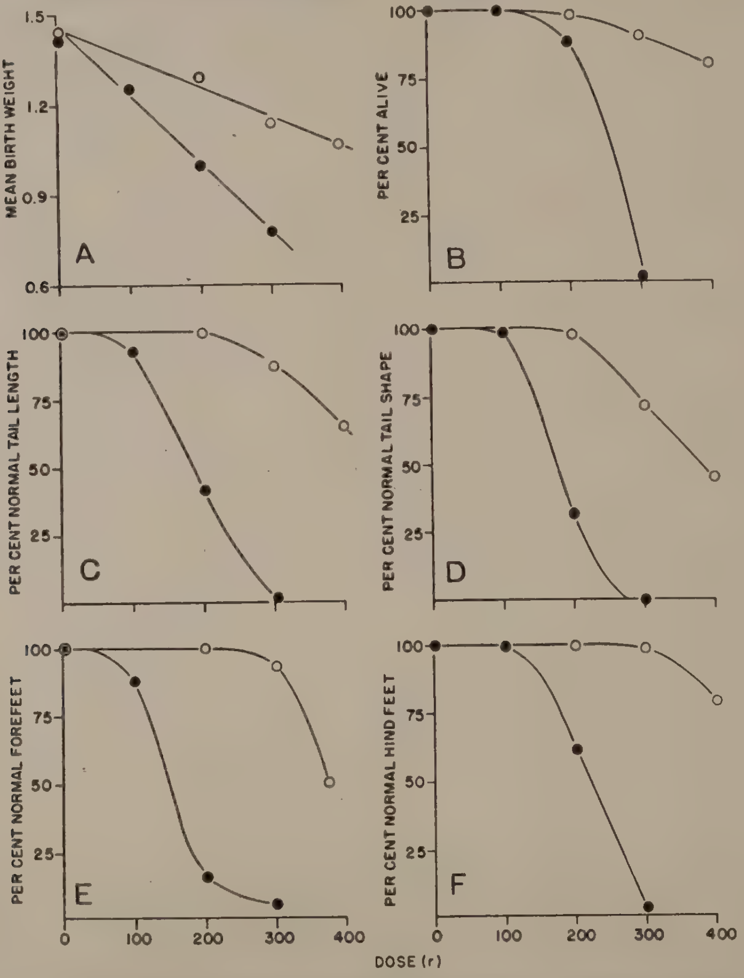

Fig. 6 Quantitative comparison between the effects of prenatal irradiation of the mouse in an atmosphere of 5% O_2 + 95% He (○———○) and in air (●———●). All treatments (0, 100, 200, 300, and 400 r) were administered on day 11½ postconception and all observations made at birth (Russell *et al.*, '51; Russell, '54). (By permission from *Radiation Biology*, Vol. I, edited by Alexander Hollaender. Copyright, 1954. McGraw-Hill Book Company, Inc.)

(A) Mean birth weight
(B) Mortality
(C) Tail length
(D) Tail shape
(E) Forefeet
(F) Hind feet

occurring in the developmental chain triggered off by the initial disturbance may, of course, be quite varied and complex. Thus, abnormalities involving an apparent *increase* rather than a deficiency of tissue, e.g., polydactyly, are still quite compatible with the view that the initial change is death of cells. Damage of this type could upset the interrelation of precursor fields in such a way as to disturb the normal limitations on growth of parts.

As already noted, one major feature of our results is that radiation causes selective damage, in spite of the general distribution of ionizations throughout the embryo. Two possible bases for differential sensitivity may be postulated: (a) The initial cellular effect of radiation, suggested (above) to be killing of cells, may be selectively determined, e.g., by differential rates of activity in different precursor regions. (b) Even among parts having equal initial cellular damage, there may be relatively less developmental effect on those still possessing great regulatory powers. Experimental results indicate that both factors are operating. Thus, in support of hypothesis (a) are the known facts of differential radiosensitivity, as demonstrated in other biological material. In support of hypothesis (b) one may recall the great regulatory power demonstrated for the early mammalian embryo as a whole during stages preceding those which yield abnormalities (see pp. 126–127). Furthermore, simple calculations (see p. 133) indicate that only if the probability (p) of affecting a cell in a precursor, and the number of cells (n) in the precursor are *both* very low, will a large proportion of animals have zero cells in that precursor affected. In cases in which 300 r gives a considerable incidence of an abnormality it may be concluded that n and/or p are not low and that, therefore, not both of these parameters can be very low for 200 r, since n is unchanged and p only proportionately smaller. In such cases, it appears quite probable that, with 200 r, cellular damage in the precursor in question is produced in a large enough proportion of the embryos for it to occur even in a small sample. There are, however, many examples (some shown in fig. 4) in which although 300 r produces a high incidence of abnormality in a certain character, no abnormality is obtained with 200 r. This indicates that regulation often follows cellular damage. In summary,

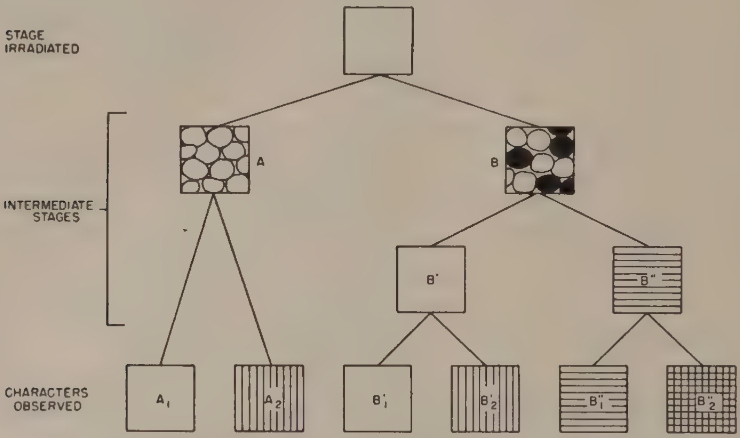

Fig. 7 Simplified diagrammatic representation of the possible pathways in the genesis of radiation-induced developmental abnormalities. It shows selectivity of early developmental damage as due to both (a) uneven distribution among precursor regions (A, B) of initial cellular damage (probably consisting of death of cells, see text) and (b) different degrees of regulatory powers of different processes (B′, B″). Thus, A_1 is normal because of lack of damage to precursors; while B′ and B_1' are normal in spite of damage to precursors. Following the initial localization (B″) of developmental damage, abnormalities may be produced in direct line of cellular descent or they may be secondarily caused. Thus, the genesis of B_1'' is directly and simply traceable to initial damage. Malformation A_2 is secondarily produced (e.g., by a condition set up by B″) in a character descended from undamaged tissue (A). The production of B_2' also is purely secondary, even though the cells ancestral to this character were damaged as a direct consequence of irradiation. Finally, abnormality B_2'' is a result of interaction between direct and secondary effects.

Open areas = no damage; ● = initial cellular damage; ▤ = developmental disturbance traceable by cellular descent to initial damage; ▥ = secondary damage; ▦ = both types of damage.

then, it seems reasonable to suppose that both postulated mechanisms (a) and (b) operate to limit what may be called the initial *developmental* effect: i.e., that there is differential killing of cells (fig. 7, A vs. B), and that the effect of a given degree of killing within a process will, in turn, depend on the degree of regulatory power of that process (fig. 7, B′ vs. B″).

In addition to damage proceeding by direct cellular descent from the earliest developmental effects of radiation, there may be secondary effects. For example, if direct radiation damage caused the inability of a primordium to perform a specific metabolic function, this would secondarily affect all processes dependent on this function for normal development. The secondary effects may be on processes which have escaped or recovered from primary radiation damage; or they may be on primordia already abnormal in direct consequence of the initial damage, in which case the secondary effects will modify the malformation produced. Most of the ideas set forth in this section are diagrammatically summarized in figure 7 to emphasize the importance of caution in deducing the initial damage from the malformation observed.

Some of the ideas discussed may be mathematically generalized as follows: if $p =$ the probability of affecting (i.e., probably killing) a potentially sensitive cell in a given precursor, and $n =$ the number of cells in the precursor, then the terms of the expansion $[(1 - p) + p]^n$ give the proportions of animals with 0, 1, 2, 3, . . . , n cells of the precursor affected. One may assume that development can proceed normally if the proportion of cells killed is 0 to x/n [hypothesis (b), p. 131], but that an abnormality will result if $(x + 1)/^n$ to all cells are affected, and that the proportion of affected cells (above the threshold) determines the degree of abnormality. The threshold x/n is different for different processes and is an expression of their regulatory powers (cf. B′ and B″ in fig. 7). The probability, p, may also be different for different processes [see hypothesis (a), p. 131]. For any given process, as p increases on raising the dose, the proportion of animals in the $(x + 1)/^n$ to 1 classes becomes relatively greater, i.e., the incidence of abnormality increases; and the mean number of affected cells, pn, increases, i.e., the average degree of abnormality is raised. It is possible that a study of dosage relationships will lead to the actual determination of *p, n,* and *x* for various abnormalities. The above binomial expansion illustrates that even with fixed values of *p, n,* and *x* for a given precursor there will be statistical variability in the number of cells that are killed. Thus, even if the material were perfectly uniform not only genetically but also with regard to developmental age, etc., there could still be variation in the end results of a given dose of radiation. This fact has not been recognized by some investigators who have felt bound to attribute observed variation to variability in biological material or experimental conditions.

When comparing the action of radiation with the action of other teratogens or of mutant genes, differences in the basis of selectivity of developmental damage should be expected both by hypotheses (a) and (b) stated above (p. 131). (a) Unequal distribution of initial cellular effect is even more probable for mutants and injected poisons than it is for radiation and may have a different basis. Thus, the distribution of injected poisons is likely to be governed by chemical affinities, as well as by gradients determined fortuitously by the portal of entry. In the case of mutants, too, it must be remembered that the gene, though present in every cell of the body, probably acts only under special conditions arising in the course of differentiation. Its action may, for instance, be limited by the conditions existing in an entire tissue (as postulated for *se* and *un,* see p. 120), while this has been shown probably not to be so for developmental radiation injury. In the case of radiation injury, the pattern of differential distribution of the initial cellular damage may be determined by some common attribute (e.g., high rate of activity) of a variety of precursor processes. (b) The pattern of further selection, as a result of differences in degree of regulatory power, among regions affected by initial cellular damage depends both on the quantity of altered cells and the quality of the change. Both may be expected to vary with the teratogen. Thus, e.g., in the case of a mutant gene, it is not unreasonable to suppose (i) that the initial cellular effect is not death and (ii) that every cell within an affected region is altered so that damage cannot be repaired even in the case of processes with high regulatory powers. It has been pointed out earlier (p. 118) that quantitative changes in radiation dose may have qualitatively different results, but with radiation, an agent whose effects are so widespread (see p. 120), the practical dose range is sharply limited by lethal action, so that, within a given region, it would be difficult to achieve an effect as concentrated as that produced by gene activity.

However, regardless of great differences between teratogens in initial effect and in the basis for selectivity, once a particular disturbance is made, subsequent development is likely to be channeled into one of a very limited number of possible routes. This may account for similarities in certain details between the end results of different teratogens. Thus, parallelism between the effects of irradiation at a certain stage and those of a given mutant gene may be profitably used to point out weak links in developmental chains and to provide clues as to time and nature of some secondary gene effects, but can, in general, not be used to draw conclusions concerning the nature of primary gene action.

SUMMARY

1. A survey and discussion are presented of the general results of a number of our experiments in which several thousand genetically controlled mouse embryos were irradiated with doses ranging from 25 to 400 r and at stages of development ranging, by 24-hour intervals, from day ½ to day 13½ postconception.

2. A high incidence of prenatal loss, due to both terminations of pregnancies and death of individuals within surviving pregnancies, is observed following irradiation during the preimplantation period. The incidence of prenatal death is decreased as the age at irradiation is advanced. With 200 r the decrease is from about 80% for treatment in early pre-

implantation stages to almost zero for treatment from day 10½ until the end of gestation.

3. Neonatal death is at a peak following irradiation on days 9½ and 10½. With 200 r, no neonatal death in excess over controls occurs in animals which were treated before day 7½ or after day 11½ postconception.

4. Decrease in birth weight follows irradiation of postimplantation stages and is greatest as a result of irradiation around day 11½.

5. Irradiation with 200 r during the period of major organogenesis, days 6½ to 12½, produces high incidences of a great number of malformations. On the other hand, irradiation with the same dose causes virtually no gross abnormalities of the types looked for at term in survivors from treatment on any one of days ½ to 5½, as well as 13½ and probably later days.

6. On the assumption that the effect of radiation is death of cells (see point 9), it can be calculated from the observed frequency of failure of irradiated one-cell stages to develop that among the survivors from irradiation of subsequent preimplantation stages were probably some in which a considerable proportion of blastomeres were killed. If this is true, then the early mammalian embryo shows great regulatory powers.

7. Within the period of major organogenesis, the developmental interval during which radiation must be applied to give the bulk of the incidence of any one malformation is usually restricted to one or two days if the dose used is not so high that saturation effects are produced. The time interval during which this dose elicits the abnormality is referred to as its "critical period." A few abnormalities have two critical periods.

8. For many abnormalities, there is probably no sharp beginning and end of sensitivity, but an increase in sensitivity leading to a peak, and then a decrease. This is shown by chronological extension of the responding period on use of a higher dose.

9. A critical period localizes chronologically the first step in teratogenesis. The hypothesis is advanced that, at the biological level, the primary intracellular effect of radiation on the embryo is the induction of somatic chromosome aberrations which result in cell-lethal action at or after mitosis.

10. Hypoxia protects markedly against radiation-induced abnormalities.

11. The maternal organism probably does not play an important intermediary part in the production of most radiation-induced abnormalities.

12. Since at any one stage of development only certain ones of the critical periods come to their peak, the results indicate that there is differential sensitivity to developmental damage in spite of the general distribution of ionizations throughout the embryo. It is postulated that two mechanisms limit the initial *developmental* damage of radiation, namely, (a) uneven distribution of the initial cellular damage (= death of cells, see point 9), and (b) dependence of the developmental effect of a given amount of cellular killing within a process on the regulatory power of that process.

13. The complex chains of processes leading to the finally observed malformations may be related by direct cellular descent to the initial developmental effects of radiation (see point 12), or they may be secondarily caused. There may, further, be interactions of primary and secondary abnormal processes. (A diagrammatic representation of points 12 and 13 is shown in fig. 7.) It is therefore not surprising that, although in the case of a number of abnormalities sensitivity is apparently linked with a high degree of activity in the primordia themselves, there are several other abnormalities for which sensitivity is not predictable on this basis.

14. The postulated chain of events between radiation and observation (points 9–13) may, then, be summarized as follows: ionizations (generally distributed throughout embryo) → chromosome breaks = *primary intracellular damage* → chromosome aberrations → failure at mitosis or aneuploidy after mitosis → death of cells = *initial cellular effect* (prob-

ably unevenly distributed) → (if regulatory power of process inadequate for given amount of damage) *initial developmental effect* → (i) observed damage related by cellular descent to initial developmental effect: (ii) observed damage caused indirectly by initial developmental effect; or (iii) interaction of (i) and (ii).

15. The results fit a mathematical generalization which assumes that a threshold proportion of cells must be affected in a precursor in order to produce any abnormality and that, above this threshold, the number of cells affected determines degree of abnormality.

16. The dose-incidence slope at the stage of maximum sensitivity is different for different abnormalities.

17. The processes that are disturbed by radiation at a given stage are more numerous than those affected even by a highly pleiotropic gene or by chemical teratogens recently used on inbred mouse material. This more widespread action of radiation exists even for doses that produce a less severe change than the gene. Occasionally, however, good parallelism in detail is obtained. The bases for differences and similarities are discussed.

18. Radiation can copy the action of prenatal environmental factors on the axial formula and related changes.

19. Using suitable characters, comparisons between genetically different groups of mice indicate that strain differences in the final visible result of a given dose of radiation are probably due to differences in developmental potencies rather than differences in sensitivity to primary radiation damage.

DISCUSSION

Chairman WRIGHT: I was struck by the rather remarkable difference between the abnormalities that you get here and certain types that I am familiar with in the guinea pig, especially cyclopia and otocephaly. These are, in general, the most characteristic types of abnormality produced by extremely early chemical treatment in vertebrates. They also often have a genetic basis, as in one of my inbred lines of guinea pigs.

L. B. Russell: In our observations at birth, we have not seen cyclopia, or any approach to cyclopia; and although we have at least two types of shortened lower jaw, this condition has in no case involved fusion of the mandibles and is, therefore, not otocephaly. We have not, however, excluded the possibility that cyclopic, otocephalic, and other types of maldevelopment exist in the large group of embryos that die early in gestation following irradiation of preimplantation stages, or in the 27% missing at birth following irradiation on days 6½–8½.

Vogel: Can you determine from your preliminary neutron results whether, for doses physically comparable to your X-ray doses, neutrons give comparable or different percentages of the same types of abnormalities?

Russell: For the stages of development studied so far, equal incidences of various abnormalities are obtained if the cyclotron neutron rep used is approximately one-fourth or one-fifth of the X-ray dose. That is, the RBE for these particular cases is 4–5.

Sheppard: It should be added that if the proportion of the dosimeter reading due to γ contamination in the cyclotron exposure was greater than assumed, then the RBE is higher than estimated.

Noonan: Would it be possible to relate the relative insensitiveness of preimplantation stages to a lower oxygen tension in the tissues during that time?

Russell: To consider the preimplantation embryo insensitive to radiation on the grounds that the survivors are free of abnormalities is a mistake that is easily made. Actually, depending on exact stage, 43–83% of the embryos receiving 200 r during the preimplantation period die before the halfway point in gestation, indicating high, rather than low, radiosensitivity. The normality of survivors is — as we have tried to show — probably due to great regulatory powers of the early mammalian embryo.

J. G. Wilson: I should like to ask Dr. Russell in what sense she used the term "critical periods." That term has been used to mean that an embryo may be undergoing some critical process at the time that a noxious agent is administered and that the process is interfered with directly. I take it that Dr. Russell does not use the term in that limited sense at all. That leads to another question. What about the malformations that were induced by radiation before there was cartilage or precartilage or any differentiation of any kind except the most rudimentary mesoderm, as is found in, for example, the ninth-day rat and I presume in about the seventh-day mouse embryo? How does this fit into your conception of critical periods?

Russell: I might define "critical period" (in the sense in which it is used by us) as the developmental moment or interval during which radiation must be applied to produce a given change at some specified stage of observation. The term "critical period" does not imply that the immediate primordium of the character malformed as a result of irradiation at a given stage was damaged at that stage. As we have shown in figure 7 of our paper, the complex chains of processes leading to the finally observed malformations may be related by direct cellular descent to the initial developmental effects of radiation, or they may be secondarily caused, and there may, further, be interactions of primary and secondary abnormal processes. However, the *initial* disturbance that touches off the chains of processes, regardless of their complexity, must occur at a certain stage (the "critical period") to give a certain end result.

As to your specific question, our view that certain specific types of malformation of the ossified vertebral column result from injury to the early primitive streak does not seem to me to be in the least inconsistent with our concept of critical periods; in fact, I should have been greatly surprised if "intersegmental jumbling" of vertebrae had resulted from a disturbance at the time of cartilage formation.

WILSON: Then such systems as the digestive tract, which I believe has not been shown to develop malformations after any treatment with X rays, do not have critical periods?

RUSSELL: We have reported gross changes at the posterior end of the digestive tract, namely, small or imperforate anus (Russell, '50). Undoubtedly, more changes could be found on histological examination, which we have not performed. Although your own thorough work has included histological observations, all of your irradiations were performed during a restricted developmental interval (day 8, 9, or 10) and it is quite conceivable that critical periods for various digestive system abnormalities occur somewhat later.

WILSON: We must always distinguish between malformation and pathological change. For radiation at later stages, sensitivity may be increased again, but after differentiation is completed it might result in a pathological change.

WARKANY: Maybe I can make a contribution to this discussion by explaining why Dr. Wilson and I argue against the indiscriminate use of the term "critical period." There is at present in the medical literature a tendency to assume that the date on which an abnormality becomes recognizable is identical with the date on which the fetus is injured. I do not think that anybody here believes that, but this has become a widespread assumption which may be of practical consequence, and we disagree with it. This may explain why we are opposed to using the term "critical period" without strict definition.

WAELSCH: I should like to ask a question about the mechanism by which the development, particularly of the skeletal system, may be affected here. You seem to trace it back to direct effects on the primordia of the structures observed. Couldn't you assume that some more general physiological process which is involved in normal skeletal formation is affected by the X rays, and that, e.g., chondrification and osssification become abnormal due to physiological disturbances?

RUSSELL: I am sorry if I left the impression that it is always the primordium of the character observed malformed which is affected at the "critical period." It is obvious that many abnormalities are probably secondary to other abnormalities (e.g., sternal malformations traceable to defects in thoracic vertebrae) and there are undoubtedly many other cases in which the final malformation is the result of as yet unsuspected chains of reactions. Initial developmental damage of radiation (B'' in fig. 7 of our paper) brought about by death of cells, may create an altered physiological condition which secondarily affects certain processes selectively (A_2, B_2', B_2'' in fig. 7). However, it seems to me very unlikely that all or even a large number of abnormalities can be explained by such a physiological condition producing a general interference with chondrification or ossification, as you have suggested. Since many severe skeletal abnormalities can be produced by irradiating long before chondrification or ossification begins, such a physiological condition would have to persist for a considerable period of time. If, on the other hand, there is such long persistence of action, then chondrification or ossification processes taking place on a number of consecutive days could be disturbed and one would, therefore, not get the clear dependence of abnormality on stage irradiated that has been demonstrated.

ATWOOD: It should be emphasized that Dr. Russell's hypothesis of cell injury involving the chromosomes and mitotic apparatus is the only one which receives any support from general experience with other biological systems. Many experiments, some of them critically decisive, have implicated the nucleus as the primary mediator of radiation damage. Effects on other cell constituents, metabolic processes, and enzymatic activities do not become important except at doses far in excess of those which permanently preclude normal mitotic activity in a majority of cells. This does not mean that X rays have a specific effect. Instead, it indicates that cells are organized in such a way that the loss of chromosome fragments, requiring one or few "hits," is usually lethal; whereas other constituents are so manifold as to require many "hits" to be exhaustively inactivated, or reduced to a threshold level. Similarly, primary radiation death of cells in a tissue must be the result of elementary processes randomly distributed in space, and of course devoid of biological specificity. It is therefore clear that every specific effect observed in Dr. Russell's material reflects an organizational property of the embryo at the time of irradiation. Whatever this property may be, the critical period represents its duration.

As Dr. Russell has pointed out, the dose required to produce a malformation according to this hypothesis will be a very strong function of the number of cells which must be inactivated. A critical period, then, might begin when the embryo will no longer replace certain cells from an undifferentiated pool, and end when the number of differentiated cells has become sufficiently large so that the dose necessary to reach the threshold for the malformation exceeds the lethal dose for the embryo.

The questions of whether the critical cells are visibly differentiated, or constitute primordia in the anatomical sense, can only be settled by independent methods.

J. W. WILSON: These bones you are talking about here are all replacing cartilage, aren't they? Is there anything that happens to the other ossifications that come later?

RUSSELL: We have numerous instances of abnormalities in membrane bones, as well as in the cartilage bones of which I have shown illustrations.

GRUENWALD: If you compare radiation, which may have latent effects on development, with any other hypothetical agent that acts only at the moment when you put it in, could it be that different effects would result?

RUSSELL: I cannot conceive of any agent with more immediate initial effects than radiation. We know that almost immediately after we press the switch on the X-ray machine, ionizations are produced in the embryo. All chains of processes, no matter how complex, are touched off by this event, and the developmental moment at which we have pressed the switch is the "critical period" for the end result observed at some later stage. In the case of some injected teratogens, on the other hand, there may be considerable delay before the agent reaches the embryo in effective concentrations and it may not be legitimate to consider the moment of injection synonymous with the moment of initial disturbance.

WEISS: I wonder whether it is not really worth while to state that we are facing here one of these perennial confusions between criteria. We can measure an effect only by the time we see it or have some other way of detecting it. It is entirely irrelevant whether we see the effect rather early or perhaps only after puberty has been able to show the defects to the gonads, which is a much longer latent period. We ought to look for the moment when the effect works and learn to trace back from our criteria.

I do not think we should make sharp distinctions between the sort of thing that is morphologically perceptible and that which is not, because the morphological abnormality is only a derivative expression of an earlier modification of the general cellular conditions, some of which become optically evident, while others do not.

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PLATE 1

EXPLANATION OF FIGURES

Skeletons of newborns

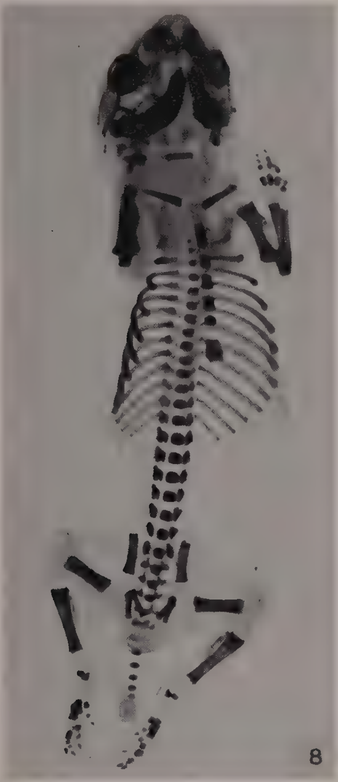
8 Control.

9 Irradiated day 7½ postconception with 200 r (mandibles, pectoral girdle, arms, and sternum removed). Note intersegmental jumbling in cervical arches and thoracic centra; fusion of ribs; non-ossification of cervical centra.

10 Irradiated day 9½ postconception with 300 r (left half of pelvic girdle and leg removed). Note reduction in number and fusion of sternebrae; extensive abnormalities in sacral and posterior lumbar vertebrae resulting in spina bifida; central constrictions in thoracic centra; thinning of ribs (e.g., No. 10, left).

11 Irradiated day 10½ postconception with 300 r (mandibles and sternum removed). Note extensive abnormalities in almost every part of the skeleton, e.g., reduction of scapulae, abnormal curvature of clavicles, fusion of humerus with radius and ulna, reduction of ilia, absence of femurs and of left fibula, reduction of tibiae and of right fibula, fusion of consecutive vertebral centra, central constriction or splitting of vertebral centra, numerous rib abnormalities.

12 Irradiated day 11½ postconception with 300 r (mandibles, pectoral girdle, arms, and sternum removed). Note cleft palate; arch-centrum fusion, angulation, breakage, and anterior displacement of ribs; bilateral loss of last rib (rib No. 13) intrapelvic crowding of vertebrae.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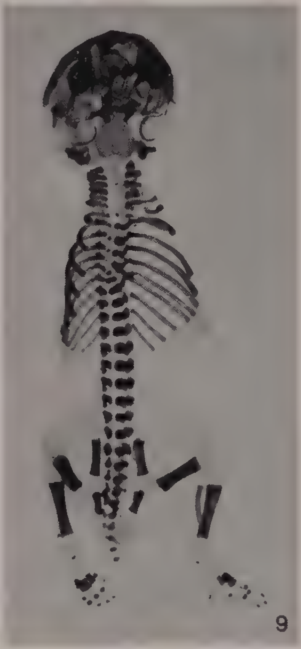
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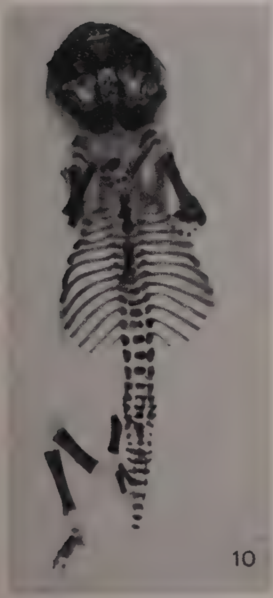
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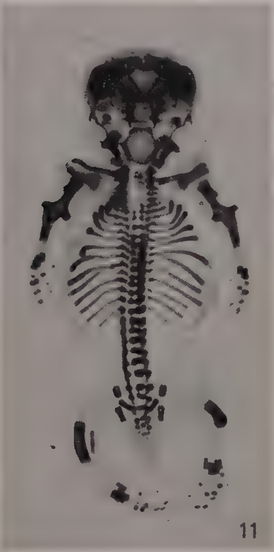
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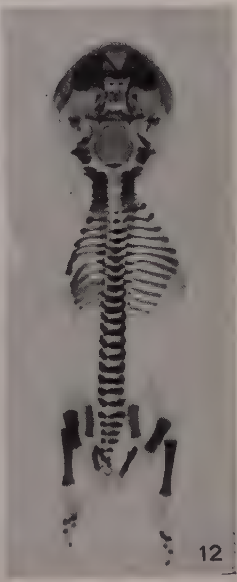
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THE EFFECTS OF IONIZING RADIATION, CERTAIN HORMONES, AND RADIOMIMETIC DRUGS ON THE DEVELOPING NERVOUS SYSTEM[1]

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ELEVEN FIGURES

During the last several years a program has been in progress in this laboratory in which various forms of metabolic inhibition are administered to adult, newborn, and pregnant animals, following which they are studied pathologically. The purpose of this study has been threefold: (1) to learn how various tissues, especially parts of the nervous system, differ from one another metabolically and how they will therefore respond differently to the agents; (2) to learn in a similar way how embryonic tissues differ among themselves, and from the adult, and how their metabolism changes during development; and (3) to correlate specific metabolic vulnerabilities of these normal tissues to the problems of cancer chemotherapy and abnormal development.

In the course of administering a wide range of forms of metabolic inhibition from anoxia and hypoglycemia to cytochrome oxidase reagents and vitamin analogues to pregnant animals, it was decided to investigate the effects of ionizing radiations on the developing nervous system. This part of the study using radiation as the "metabolic reagent" was prompted by the plight of a microcephalic girl in a neighboring institution, whose condition was alleged to be due to irradiation of her mother's pelvis for a cancer when she was an early fetus. At that time there was still much confusion in some medical circles regarding the effects of radiation on the nervous system of the embryo in spite of clinical and some experimental studies. What was especially not known was how radiation produced its effects. This problem was attacked by learning as much as possible about the mechanisms by which malformations and errors in development are brought about, and this presentation will be concerned with that aspect of the problem. Some of this material has been previously published (Hicks, '52, '53a, b, c), but most of the experiments to be illustrated are new.

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RADIATION EXPERIMENTS

In more than a thousand animals, it was found that the primitive differentiating cells of the nervous system are probably the mammalian cells most easily destroyed by ionizing radiations. In fetal and newborn rats and mice (and other animals) substantial numbers of these cells become morphologically necrotic in less than 4 hours after as little as 40 r (200 kv, 10 ma, 25 cm, 3 mm Al filter) is given to the whole body of the mother or the newborn. The effect is a direct one of radiation on the cell, there being no evidence that secondary humoral, maternal, vascular, or other factors are responsible.

In previous reports these radiosensitive cells have been collectively called neuroblasts in the sense of being blast or undifferentiated forms of future neural cells, both neurons and glia, yet one step removed toward maturity from the primitive germinal epithelium or neurectoderm. They are sufficiently primitive for it to be difficult to determine which cell will become one of the glia and which a neuron, except in some regions where the ultimate adult cellular configuration indicates what was the nature of the precursor cells. The term neuroblast ("neuro," pertaining to nervous tissue and ‘‘blast,’’ a sprout or germ) is etymologically sound for the primitive cell that has not yet showed its colors. It has also been used to indicate on the one hand a very young neuron or one beginning to take on the characters of the adult cell. The terms medulloblast, indifferent cell, intermediate cell, or mantle cell, are not useful here for a number of reasons. The most practical term for these radiosensitive neural cells would seem to be primitive neuroblasts (precursors of neurons) and spongioblasts (precursors of neuroglia), for they must later become either neurons or neuroglia. Obviously all stages of differentiation are seen from the most primitive form that results from mitotic division of the neurectodermal cell to a young neuron or glial cell (Flexner, ’50; Hamburger and Levi-Montalcini, ’50; Weiss, ’50).

In the forebrain of the early third trimester mouse or rat fetus these growth relations are well exemplified. The cells lining the lateral ventricles, which show enormous numbers of mitotic figures, are the primitive germinal epithelium or neurectoderm and the compact zone of cells adjacent to these are the primitive neuroblasts and spongioblasts. The latter in hematoxylin and eosin preparations are small and round to oval in shape; they have scant cytoplasm and a compact granular nucleus. Mitotic figures in this zone are rare even when colchicine is given experimentally to detect them more easily. Distinction between those that will become glia and those that will become neurons in the lateral periventricular zones is not possible with ordinary preparations but such radiosensitive cells in the white matter at the angles of the corpus callosum with the lateral ventricles seem to be oligodendroglia precursors. In the outer cerebral cortical zone the majority will become neurons and later in the outermost zones of the cerebellar folia many of these cells will become granule-layer neurons. The early neural plate and the early central cells of the spinal cord and brain stem are considered to be neurectoderm. As term approaches the amount of neurectoderm diminishes; in a late fetus ependymal cells have come to line the medial walls of the lateral ventricles, and neurectoderm with an adjacent zone of radiosensitive primitive neuroblasts and spongioblasts may be seen laterally. This hierarchy of cells in respect to radiosensitivity is illustrated in figure 1, where necrotized radiosensitive cells, undamaged neurectodermal cells, ependymal cells, and neurons are seen.

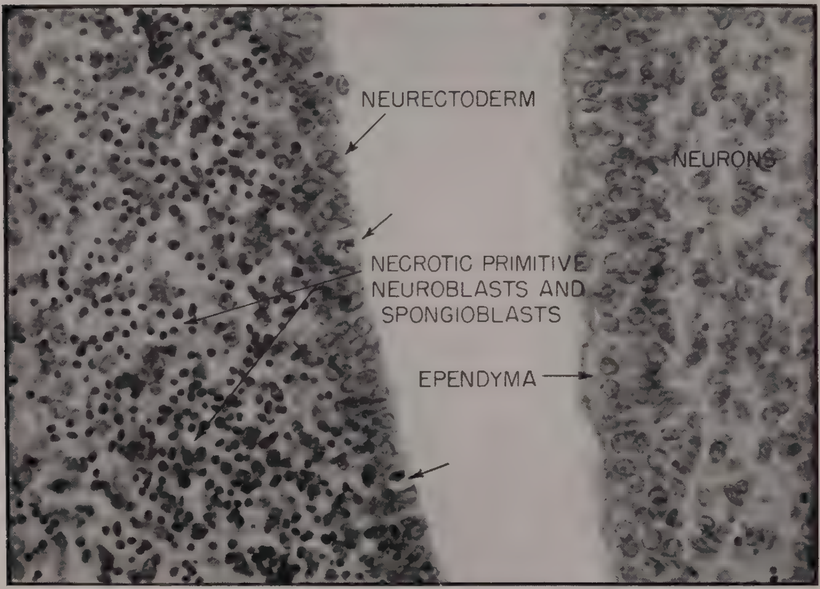

Fig. 1 The hierarchy of cells in the developing rat brain. Acute radiation necrosis of primitive neuroblasts and spongioblasts and sparing of neurectoderm, ependyma, and neurons (H & E, × 600).

The nerve cell may therefore arbitrarily be considered to pass through four stages of development: neurectoderm, neuroblast, neonatal neuron, and adult neuron. (Present experimental evidence suggests that glial cells as well as many other cells in the body pass through analogous stages of development in so far as response to radiation is concerned.) The primitive neuroblast, as noted before, is the most radiosensitive and mitoses in zones of these cells are rare. Neurectoderm, an actively mitotic cell, is not destroyed until some 600–800 r are given, and the neurons of the newborn and adult animal may withstand several thousand roentgens. In our experiments with newborn, young, and adult animals, acute necrosis of rare neurons appeared only after 1500 r and relatively few more were destroyed by 15,000–20,000 r. Oligodendroglia and ependymal cells seem to follow a somewhat similar pattern, and only a fair scattering of oligodendroglia are destroyed by single doses of radiation well above 1500 r. This striking differential response to radiation is demonstrable from the ninth day of gestation into young adult life, so that it seems to be a cellular response that is largely independent of the animal's age of intrauterine existence.

It will be immediately evident that, because this hierarchy of cells is shifting in position and balance throughout fetal and neonatal life, a broad time-spectrum of malformations could be produced by irradiating at different periods of development. This spectrum of reproducible malformations, as far as it has been studied, has been presented elsewhere in detail and is summarized in the timetable of table 1. More recent (unpublished) experiments have added considerable detail to this sequence of events and have been concerned especially with correlating the exact site and the mechanism of injury in the embryo, fetus, or newborn with the subsequent resulting malformation. This has been done by removing whole litters of developing animals at intervals of 2, 4, 8, 12, and 24 hours or at daily intervals after irradiation to follow the pathologic sequence of events leading to a given pattern of abnormal development.

Also in a recent series of experiments animals were irradiated on certain days postcoition and then 4 hours later, which allows sufficient time for radionecrosis of appropriate cells to become morphologically unequivocal, one horn of the uterus was removed for histologic study of the embryos or fetuses. The fetuses in the other horn were allowed to develop and were studied later. This method makes possible a good estimation of the age of the fetuses, the site of the injury, and the resultant malformation all based on one litter.

In figure 2 some "bright lines" in the aforementioned spectrum are shown. Important sites of vulnerability to radiation injury in the nervous system are emphasized in black and the salient features of the malformation from radiation at that time are indicated. For example, cord and brain stem deformities are outstanding when irradiation is given on the eleventh day, whereas certain cerebellar deformities occur at much later periods.

TABLE 1

Timetable of radiation malformations in rats and mice

DAY OF RADIATION	NERVOUS SYSTEM	OTHER
0 to 9 days	No damage	
9th day	Anencephaly (extreme defects of forebrain)	Severe head defects
10th day	Forebrain, brain stem, or cord defects	Skull, jaw, skeletal, visceral defects; anophthalmia
11th day	Hydrocephalus, narrow aqueduct, encephalocele; cord, and brain stem defects	Retinal, skull, skeletal defects
12th day	Decreasing encephalocele; microcephaly, porencephaly	Retinal, skull, skeletal defects
13th to 21st day	Microcephaly; bizarre defects of cortex, hippocampus, callosum, basal ganglia, decreasing toward term	Decreasing skeletal defects
Neonatal period	Cerebellar defects (beginning about 14th day of gestation to about 3 weeks after birth); cerebral defects decrease	Occasional stunted growth

A crude but very helpful analogy in understanding these processes may be had by imagining what sort of a building would result if in one case some bricks of the foundation were destroyed early in construction or if, in another case, the top floor was damaged late in construction. Provided that

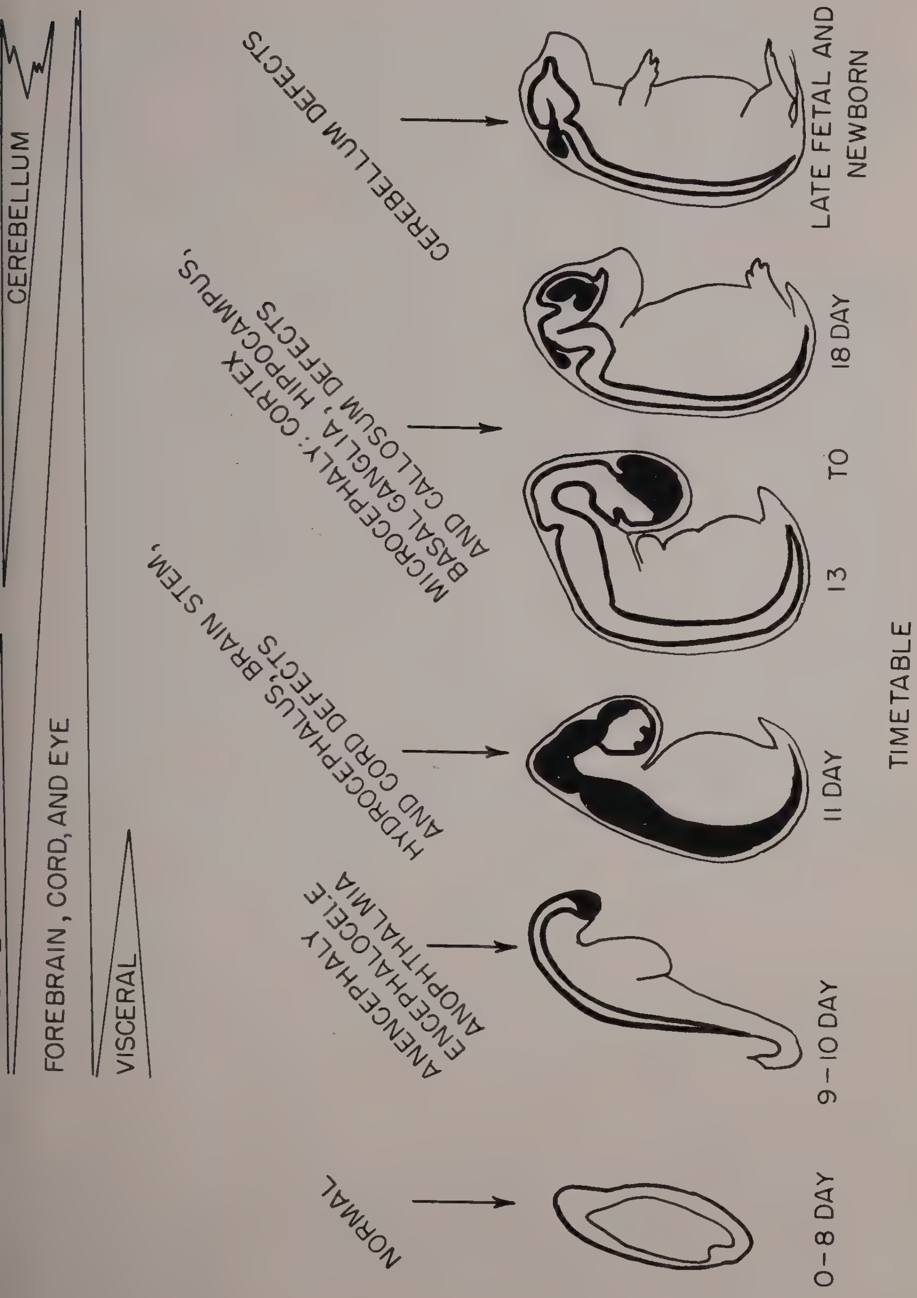

Fig. 2 Radiation malformations

the buildings had to be finished, the "malformed" structure that had its foundation damaged would take one form, the other would be quite different, and both would differ from the normal building. Such architectural patterns could for practical purposes be reproduced over and over again by interrupting construction at a given time.

Table 1 and figure 2 are based on what happens when 100–400 r are given to the total body of the pregnant and newborn rat. Most of these findings have been duplicated in mice, and a number of animals have been included in each day of the gestational period. Rats and mice have a gestation period of about 3 weeks. In spite of the inherent difficulty of timing gestation, and the fact that up to the ninth day there may be substantial "age differences" among members of a given litter of embryos, these malformative patterns have been remarkably uniform within limits. These limits, however, have certain practical and theoretical aspects. Four hundred roentgens approaches the range where maternal death may sometimes kill off a litter of embryos any time during the first 8 days, and sometimes later. Sometimes one or two fetuses may survive to show severe overall growth deficiencies. Thus the middle range has been the most useful for studying the selective action of radiation. Below 100 r damage is less spectacular and consequent degrees of malformation are less. When 600–1000 r are given, fetuses and mother may survive several days, but the long range results of such whole-body irradiation are not possible to achieve. Notwithstanding, the generalization may be made that the basic pattern of radiation malformations is dependent on the time of irradiation, and the severity is related to the dose. For example, on the twelfth day the upper parts of the cerebral hemispheres are especially vulnerable. The differences between the effects of 100 and 400 r at this time lie, essentially, in the number of primitive neuroblasts and spongioblasts destroyed in the vulnerable regions of the cerebral vesicles. The region of the brain principally affected is nonetheless the same.

Inspection of table 1 and figure 2 shows that the nervous system is not the only system affected. As noted, other tissues and cells may pass through a radiosensitive stage similar to that of the primitive neuroblast. By way of example, irradiation on the eleventh day leads to hydrocephalus, narrow aqueduct, underdeveloped brain stem, somewhat deficient cerebral mantle, and cord defects. Necrosis of the radiosensitive cells in the neuraxis is especially notable in the brain stem and cord at this time (fig. 3). Severe deformities of the spine also occur at this time — the vertebrae fail to segment and the vertex of the skull is deficient. These features in somewhat exaggerated form, resulting from a fairly high dose of radiation (600 r), are seen in figure 4. Necrosis of mesenchymal cells in the skull-to-be and developing somites of the spinal region are often not demonstrable in the dose range usually employed in these experiments, but at

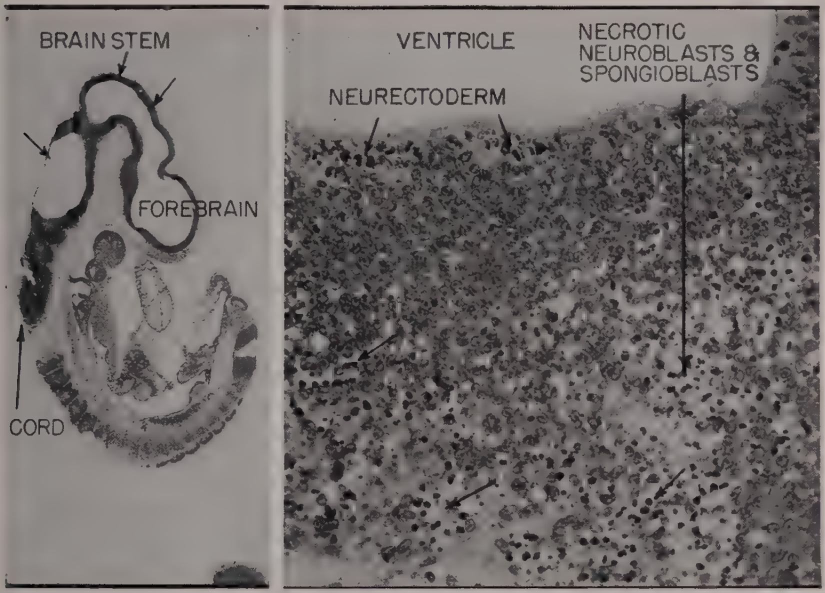

Fig. 3 Acute necrosis of primitive neuroblasts and spongioblasts in brain stem and cord of a rat fetus following irradiation on the eleventh day (H & E, × 600). Normal 11-day fetus at left for orientation.

higher doses they may be seen in sites corresponding to later malformation. Of course the volume of critical cells whose growth would have to be interrupted in these mesenchymal and preskeletal sites to produce the unsegmented vertebrae, or the skull of an encephalocele, would be small — a very little damage would go a long way. The relation of necrosis to later malformation is well illustrated in the so-called anencephalic pattern of the ninth day. The severe upper head deformity or the malformation of the face and jaws is seemingly out of proportion to the number of damaged cells, but a very little injury at this period does obviously alter the formation of the whole upper head, not to mention the brain. In figure 5 necrotic cells in the early differentiating neural folds are seen 4 hours after irradiation on the ninth day. Some necrotic primitive mesenchymal cells are also evident.

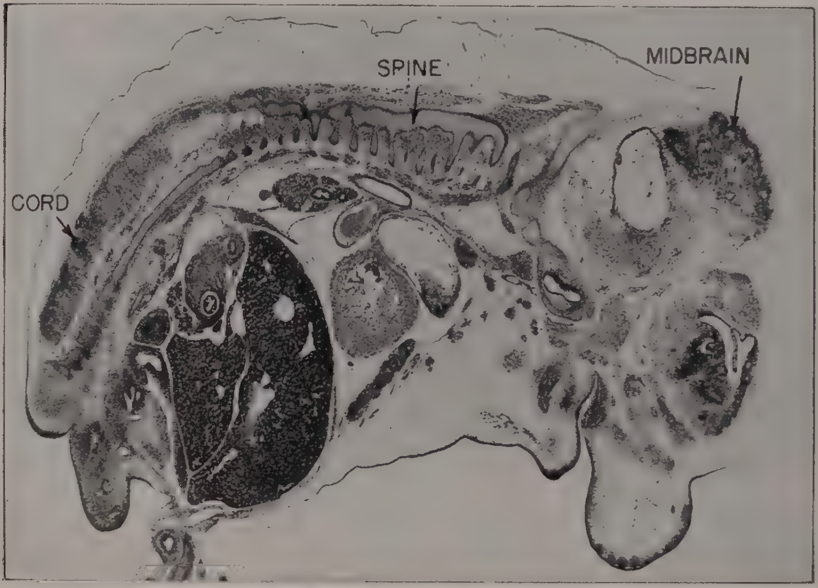

Fig. 4 Severe malformations in a rat fetus of about 16 days irradiated heavily (600 r) on the eleventh day (H & E, low power).

In the nervous system the malformations are chiefly initiated by a loss of building blocks, there being no presently

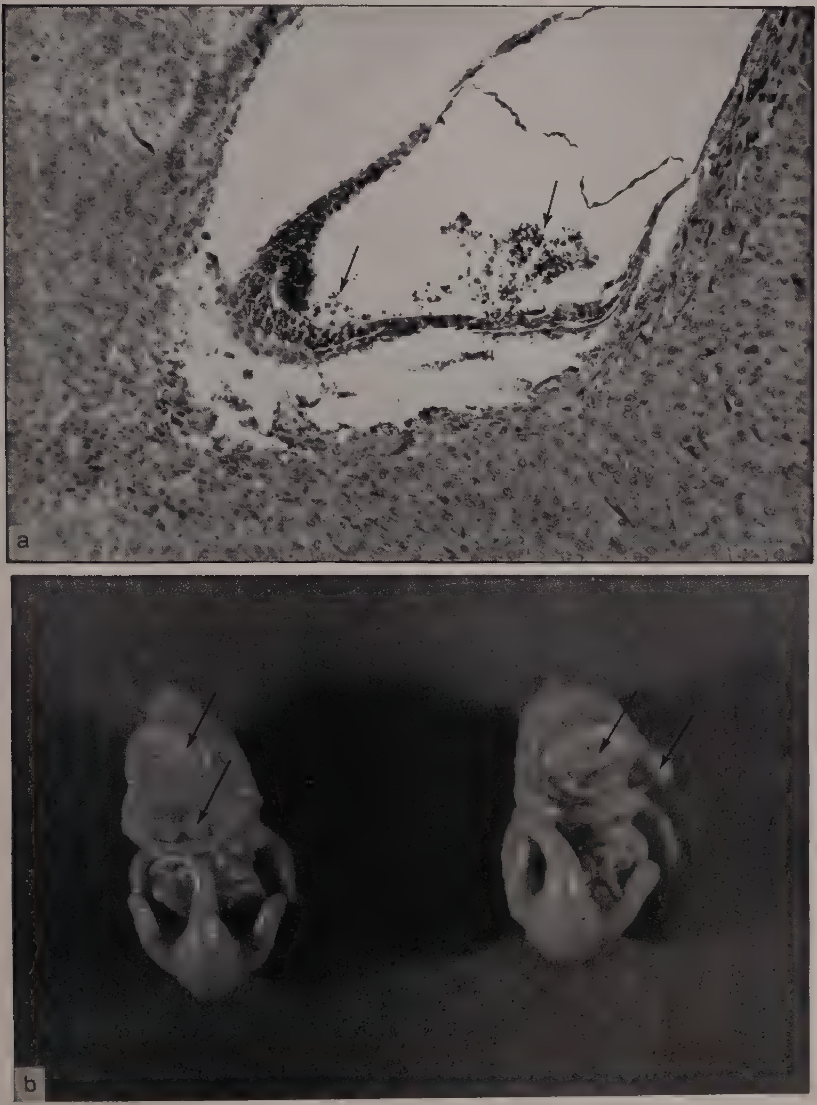

Fig. 5 (a) Radionecrosis of differentiating cells in the anterior neural folds 4 hours after irradiation of a rat embryo on the ninth day (H & E, × 400). (b) Gross malformation of forebrain and face of litter mates of (a) 7 days later.

measurable evidence that other neural cells are sublethally injured by radiation so that they will grow abnormally. That neurectoderm is not primarily affected by radiation is suggested by its taking part in the development of malformations only in sites where necrosis of primitive neuroblasts and spongioblasts occur. In other organ systems this predominantly ''all-or-none'' initiating process is difficult to demonstrate, and altered growth without actual necrosis is still a process not excluded. Further experiments in this area are being carried out to learn as much as possible about the relative importance of three factors that bear on this aspect of abnormal development: (1) actual destruction of building blocks by radiation, (2) alteration of the growth potentials of building blocks by direct action of radiation, and (3) secondary alterations of growth, for example, the effects of abnormally growing mesenchyme on the growth of the brain, or the effects of necrotic neuroblasts on the subsequent growth of neurectoderm and surviving neuroblasts.

When a 2-week-old rat or mouse fetus is irradiated the first evidence of malformation is seen in 24 hours and it is well developed in 48. Rosettes and clusters of primitive neuroblasts and spongioblasts, usually with a core of rapidly mitotic neurectodermal cells, develop (fig. 6). These rosettes with a core seem to take origin from existing neurectoderm, for the central canal or ''miniature embryonic ventricle'' of this structure is usually connected to or close to the normal canal or ventricle. However, in later fetuses, where rosette formation develops to a lesser degree, primitive neuroblasts and spongioblasts also tend to cluster and the resulting neurons in the adult animal keep this bizarre clustered and rosetted architecture. Thus the clustering tendency seems to be an inherent growth characteristic of developing nervous tissue. The characteristic is observed in normal developing sympathetic ganglia, malformed retinas from other causes, tumors of the nervous system, and in fetal animal brains damaged by certain ''radiomimetic'' drugs such as nitrogen mustard.

Some of the late patterns of malformed brains that result from radiation are shown in figures 7 and 8. In figure 7 a normal brain and one damaged during the third trimester of development (sixteenth day) are compared. The normal brain, incidentally, is that of a rat whose mother was given 400 r total body radiation on the eighth day of its gestational development, and serves to illustrate how relatively resistant the embryo is in that period. Figure 7 shows the brains of adult animals. Those that were irradiated on the seventeenth day have small forebrains and less bizarre cerebellums than their cousins that were irradiated near term. In the latter, cerebral malformation was relatively less, but cerebellar abnormality was substantial. The internal configuration of these brains varies with the time, of course, as has been outlined elsewhere, but the general gross descriptive term used is "microcephaly."

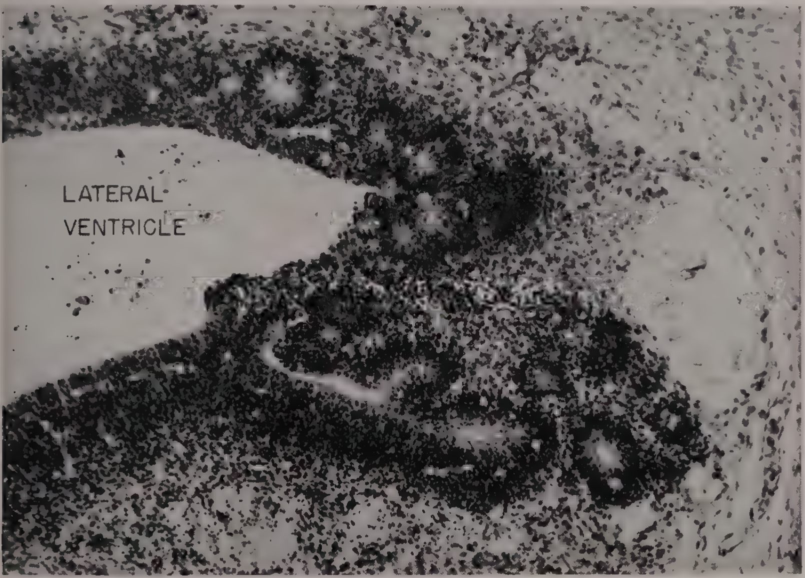

Fig. 6 First evidence of malformation in the brain following irradiation of a rat fetus of about 14 days (H & E, × 200).

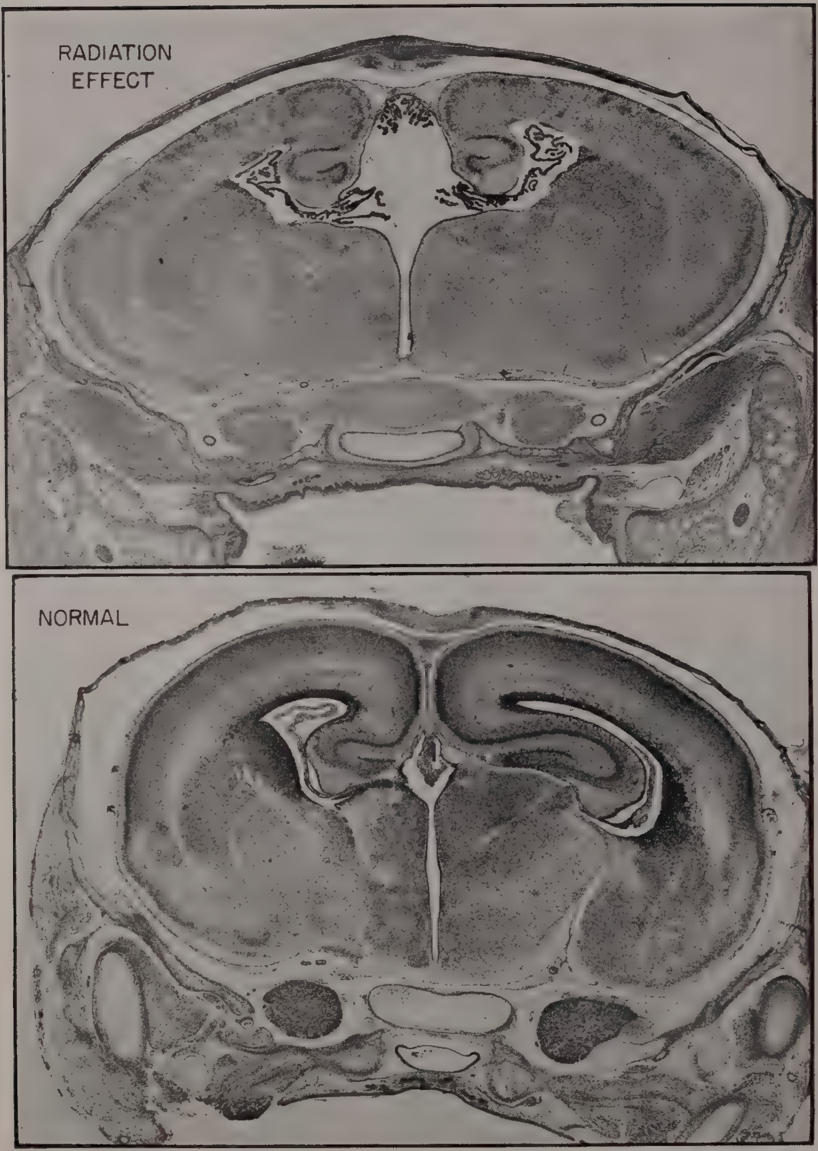

Fig. 7 Malformation of the brain of a young rat that was irradiated *in utero* on the sixteenth day. The normal brain is that of a comparable young rat irradiated on the eighth day of gestation with 400 r (H & E, low power).

The viability of developing animals irradiated at different periods varies. Other investigators have given this considerable attention. The dosage factors have been noted. Anencephalics and animals from the 10- and 11-day periods probably could not survive, and the mother animal eats them unless they are taken just before birth or isolated at birth. Some 12-day animals, and animals irradiated from the thirteenth day on, can survive, and some from each of these days have been allowed to grow to adulthood. Their behavior varies with the defects present, but detailed neurologic studies have not been carried out.

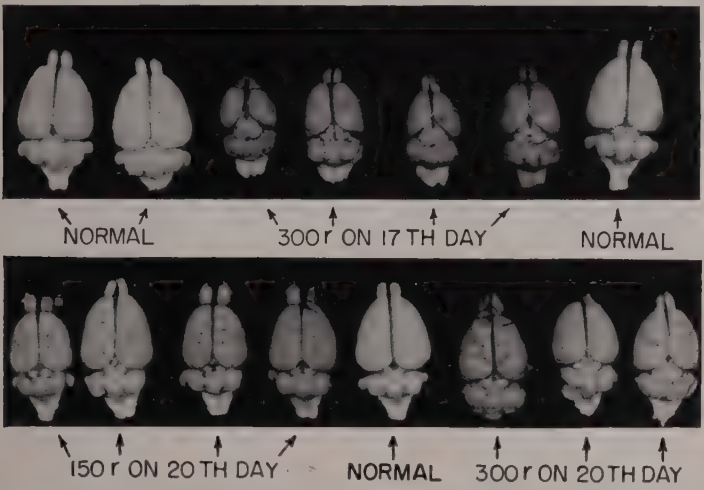

Fig. 8 Radiation microcephaly. The 17-day animals have very small forebrains and the cerebellar folia are irregular. The 20-day animals have less forebrain deficiency and the cerebellums are malformed.

CRITICAL PERIODS

Although the time relations for maldevelopments of the nervous system resulting from radiation would seem to be clear cut in these experiments, the concept of critical periods has been used in different ways by different investigators (Adelmann, '25; Russell, '50; Wilson, '53). In these experiments a dose of 200 r will in the first 8 days produce no malformations; late on the ninth day the head will be grossly malformed, the body and viscera will be normal; and so on through the series of deformities shown in figure 2. A particular cerebellar malformation will result from irradiation in a given part of late gestation, but not at any other time, because certain parts of the cerebellum are developing in late gestation and neonatal life. The intrinsic pattern of the cerebellar malformation also depends on time; speaking broadly, radiation during late gestation affects chiefly the development of the gross cerebellar folial configurations, while postnatal irradiation affects the finer cellular architecture because these respective characteristics are being formed at those times.

This does not exclude the possibility that specific malformations of the cerebellum could result in other ways than from radiation. As a hypothetical example, irradiation of a male rat might alter a chromosome in one of its gonadal germ cells which would express itself as a cerebellar maldevelopment in one of its descendants. It would then be difficult to say whether the ''critical period'' for this malformation was at the time of irradiation of the germ cell, or at any arbitrary stage in embryonic development when the sequence leading to cerebellar genesis was in progress, or at the time when the cerebellum failed to appear. Also it may be possible to find metabolic inhibitors that damage neurectoderm and produce a whole series of malformations unlike those resulting from ''radiomimetic'' agents. Such agents have not been uncovered to date.

EXPERIMENTS WITH OTHER AGENTS

Some knowledge of how changing radiosensitivity of a tissue in a fetus or embryo may be related to developmental metabolism has been gained by comparing the effects on them of other metabolic inhibitors. The advantages and pitfalls of giving metabolic inhibitors to living animals to learn about specific tissue metabolism has been discussed elsewhere, but some aspects may be included here.

The primitive neuroblast is concerned with rapid nucleic acid and protein synthesis, and seems to accomplish this at least in part in association with a system of sulfhydryl enzymes. Such enzymes are believed to be vulnerable to radiation because radiation produces oxidizing agents in the cell water. The highly selective histologic effects of radiation on the developing nervous system can be closely mimicked by nitrogen mustard and triethylene melamine, and to a lesser extent with large doses of cortisone (and some other steroids) and sulfhydryl reagents such as mapharsen, chloromercuribenzoate, and iodosobenzoate. The latter sulfhydryl reagents are synergistic when given together in appropriate fractional dosage, which lends added support to the idea that sulfhydryl compounds and enzymes play a key part in the protein synthetic reactions of differential growth. Aminopterin also damages neuroblasts, presumably by blocking a phase of nucleic acid metabolism. On the other hand, the radiosensitive neural cells are resistant to acute anoxia and hypoglycemia and to agents which acutely interrupt aerobic metabolism, when compared to adult neurons.

This resistance of the developing animal to acute anoxia and processes that interrupt the aerobic breakdown of glucose is an interesting and complex problem. If infant mice and rats are placed in pure nitrogen, they survive 40 to 50 minutes; and if revived in air and allowed to survive, they almost never show irreversible damage to any tissue, and then rarely only a few neurons are damaged. The whole body metabolism appears to slow down, but some source of energy is obviously used up. Primitive neuroblasts and spongioblasts are similarly resistant. The source of anaerobic energy is obscure. Some glycogen stores are used up during the asphyxial period, and insulin hypoglycemia or fluoride will shorten anaerobic survival. Iodoacetate, in proper dosage, said to be specific for phosphoglyceraldehyde dehydrogenase, abruptly halts survival of the animal in nitrogen but will permit the animal to survive in air. When insulin hypoglycemia is induced or a sublethal combination of iodoacetate and asphyxia are given, it is the neonatal neurons, especially of the brain stem, that are selectively damaged. The radiosensitive neural cells are again unharmed by these processes. Thus the anaerobic survival of neonatal neurons seems to be through a pathway of anaerobic glycolysis. It is presumed that this also requires energy-rich phosphate compounds formed during aerobiosis. The source of anaerobic energy of the radiosensitive cells is unknown, but adenosinetriphosphate or phosphocreatine might be sources. The fact that cortisone and fluoroacetate damage both neuroblasts and neonatal neurons (and to a lesser extent adult neurons) leads to the speculation that both may be interrupting energy-rich phosphate compound metabolism — cortisone perhaps by preventing its coupling to glucose breakdown and protein synthesis, and fluoroacetate by preventing the formation of such compounds in the Krebs cycle.

The changing responses of the developing nerve cell, *in vivo,* under experimental circumstances, to a variety of metabolic inhibitors is summarized in table 2. X radiation is listed as negative in the adult neuron column because only very large doses do acute damage and increasing dosage does not substantially increase this effect. Thus differences, not absolute measures, are implied.

The interpretations of such a tabulation are complex, but they serve to show that during development the nerve cell passes through certain distinctive stages in which the cell metabolism and vulnerability to noxious agents may vary in remarkable degree.

From table 2 it may be surmised that certain agents acting acutely could produce developmental malformations of the nervous system and others would fail to do so. This is borne out by experiment. Acute hypoglycemia will produce total death of the fetus or the mother, or the newborn animal, by damaging neonatal or adult neurons before it will measurably embarrass the neuroblasts and spongioblasts. Such altered growth as it would cause would be entirely different from that caused by radiation. In contrast, nitrogen mustard and triethylene melamine mimic malformations produced by radiation (fig. 9) but the ''margin of safety'' of these agents as experimental tools for producing developmental defects is less than with radiation. That is to say, they are not quite as discriminating as radiation in their selective action on the radiosensitive cells; for damage to maternal, fetal, or newborn bone marrow is comparatively more easily accomplished than with radiation. Cortisone mimics radiation effects on the neuroblasts but it also damages neonatal neurons and skin appendages, stunts general growth, and acts especially on certain phases of skeletal development (fig. 10). The generally small brains and slight architectural disturbances of the cerebral cortex that characterize the late cortisone malformation cannot be accounted for entirely by the numbers of neuroblasts and neonatal neurons that are destroyed (fig. 11). Probably some of the differences in histologic effect between cortisone and radiation given at birth lie in the duration of action. Radiation is immediate, while cortisone, though it does produce selective, severe acute cell

TABLE 2

Differences in response to acute metabolic injury

AGENT	NEURECTODERM	NEUROBLAST	NEONATAL NEURON	ADULT NEURON
Anoxia, azide, cyanide	—	—	—	+
Hypoglycemia	—	—	+	+
''Radiomimetic'' drugs and sulfhydryl reagents	—	+	—	—
Cortisone	—	+	+	±
X radiation	—	+	—	—
Fluoroacetate	—	±	±	±
Iodoacetate	—	—	—	—
Iodoacetate plus anoxia	—	—	+	

+ indicates cells selectively vulnerable (necrotized) to the agent.
— indicates resistant cells.
± indicates cells where acute damage is slight.

damage, is inevitably prolonged in its effect by being slowly absorbed from the site of injection. Preliminary experiments using repeated doses of radiation over a period of 3 or 4 days on newborn animals have shown a pattern of abnormal growth similar to the cortisone effects in several respects. ACTH produces acute brain damage in late fetuses and newborn like that of cortisone, but unless repeated doses are given, skin and skeletal changes do not occur.

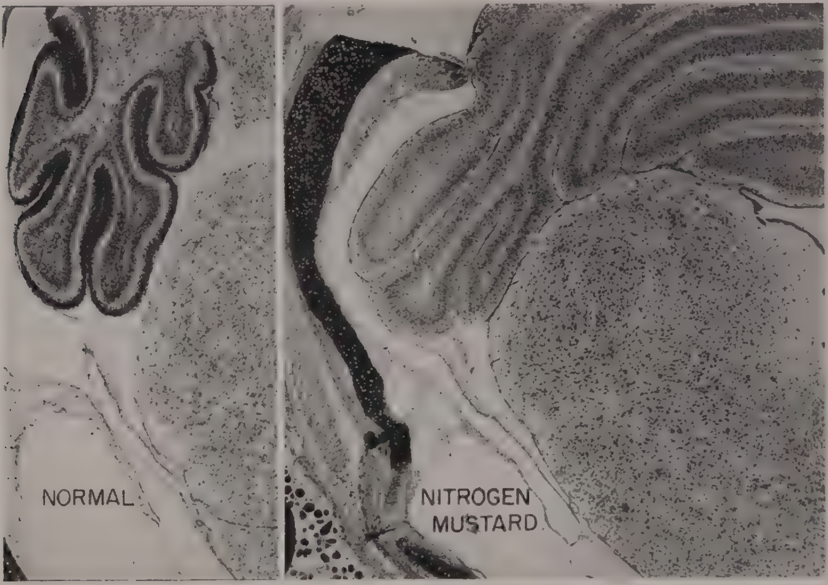

Fig. 9 Early malformation of the cerebellum in a young mouse that received nitrogen mustard at birth. This is indistinguishable from the pattern caused by radiation (Thionin, low power).

Other prenatal influences must be even more complex. Whether a virus, a metabolic reagent, a hormone, or a chemical reagent gets to the fetus across the various tissue barriers is a governing factor. How specific it is is another factor — radiation has a "wide margin of experimental safety," speaking pharmacologically, between fetal and maternal damage, whereas maphrasen (a sulfhydryl reagent) has a narrower one, and it is more difficult to produce specific effects with such a less specific agent.

The duration of action of the agent is another factor and is one of the most difficult variables to reckon with in this type of experimental work. It is inevitable that comparisons have been made of agents whose actual durations of action can only be surmised. Prolonged partial anoxia of definite duration has been shown to damage fetal cells, for they ultimately depend, like all mammalian tissues, on oxygen for energetic processes. With prolonged partial anoxia, the differences in vulnerability between fetal and adult tissues becomes considerably lessened; with acute anoxia the difference is strikingly great, the fetus escaping entirely as has been noted.

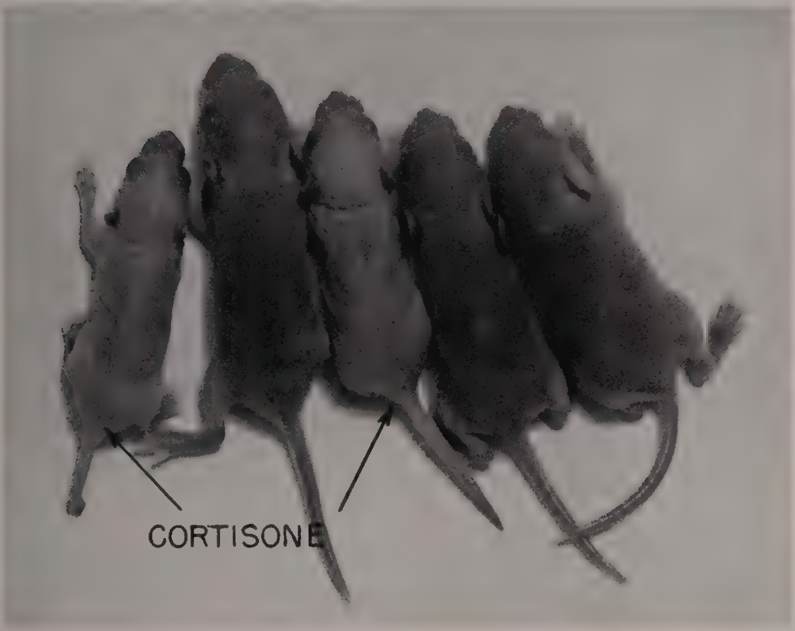

Fig. 10 Effects of cortisone at birth. The smaller, hairless mice received a large dose of cortisone at birth. They already have skeletal, skull, and brain abnormalities after a few days. The others are normal litter mates.

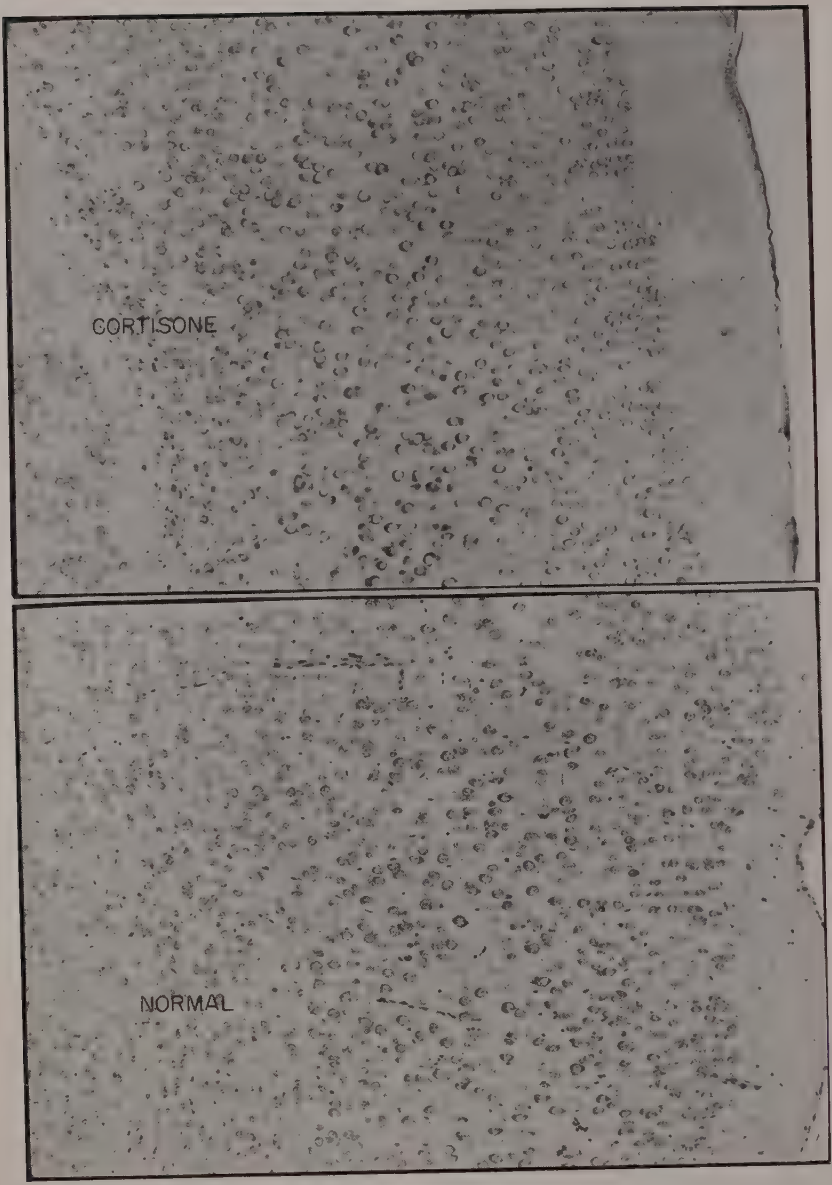

Fig. 11 Cerebral cortex of a 3-week-old mouse treated with cortisone as in figure 10. Despite the earlier cortisone damage, the abnormality is slight and manifested by a narrowed cortex (Thionin hematoxylin, × 400).

What then do all these experiments mean in terms of prenatal influences on development? They indicate strongly that the metabolic makeup of the cell is a key factor in the determination of its liability to injury, and that as the cell passes through its stages of development it may change its vulnerability to noxious agents in striking ways. From the broader view, that of considering the changing cells within the developing total animal, it may be said that a malformation of the types described here is a product of two factors: the nature of the agent and the time of injury.

SUMMARY

Ionizing radiations at low doses selectively damage the primitive neuroblasts and spongioblasts in embryo, fetal, and young animals; and, experimentally, a series of reproducible patterns of malformations of the nervous system can be achieved based essentially on the destruction of these building blocks. The patterns range from anencephaly through hydrocephalus to various kinds of microcephaly and cerebellar defects. With the effective dose of radiation remaining constant, the pattern of malformation is determined by the time during development at which the radiation is given.

''Radiomimetic'' drugs such as nitrogen mustard and triethylene melamine, and certain sulfhydryl reagents selectively destroy the radiosensitive neuroblasts and spongioblasts. Aminopterin mimics this radiation effect. Varying degrees of success in producing malformations have accompanied their use, but the selectivity of action of the sulfhydryl reagents has been less than that of radiation. Cortisone, which imitates the radiation effect on the fetal brain, also damages more mature neurons and causes skin and skeletal deformities.

The patterns of malformation that result from the action of various noxious agents on developing mammals are largely determined by the product of two factors: the character of the agent and the time of injury.

DISCUSSION

GAULDEN: The cells described by Dr. Rugh as being pycnotic and those described by Dr. Hicks as being necrotic appear to me to be similar. These two terms, however, imply different fates of the cell. A pycnotic cell is one in which the chromatin fuses into one or several masses which stain intensely. Some pycnotic cells are capable of recovery and of further division. A necrotic cell, on the other hand, is a cell which has died.

Dr. Hicks, do you think it possible that, in your material, some of the cells which disappear are phagocytized in a damaged (pycnotic) rather than in a necrotic condition?

HICKS: Dr. Rugh and I have discussed that pycnosis is a term in pathology that denotes compactness or shrinking of a cell, especially its nucleus. It is a purely descriptive term and does not indicate much about the state of the cell, and it certainly does not imply necrosis. This is a very important distinction. In our experiments the whole chain of events leading to developmental malformations is initiated by necrosis of certain cells. Mere pycnosis has not been related to this process. We have not seen phagocytosis of living cells, only dead ones.

GAULDEN: I should like to comment on the question of the role of mitotic inhibition in the production of embryonic abnormalities. In our laboratory the effects of radiation on mitosis have been studied by making direct observations on living cells *(in vitro)* of the 14-day-old grasshopper embryo. A dose of 200 r of X rays reduces mitosis to zero within an hour of treatment, and this complete inhibition of mitosis persists for several hours. Recovery is slow — mitotic activity does not return to the normal level until some hours after treatment.

Fourteen-day-old embryos given 200 r of X rays will reach the stage of hatching at the same time (ca. 42 days from time eggs laid) and in the same numbers as their untreated sisters. The young nymphs that develop from these irradiated embryos show no external morphological abnormalities and are not unlike their control sisters in behavior. They have not been examined for histological abnormalities.

Thus we see that a dose of X rays which produces what would be termed "drastic" mitotic effects in grasshopper embryos does not affect the over-all rate of embryonic development or cause gross morphological abnormalities in the grasshopper. (A dose of 500 r of X rays is 100% lethal.) These results indicate that extreme caution must be exercised in attributing embryological abnormalities to mitotic effects.

BILLEN: Have you noticed any changes more subtle than the gross changes reported for these cells? I ask that because it has been shown in certain microbial cells that material such as adenosinetriphosphate as well as other 260 mμ absorbing compounds will leak out of X-irradiated cells.

HICKS: So far, the radiosensitive cells respond in an all-or-none way. They are either destroyed or they seem to be unaffected. We are starting some histochemical studies to learn whether nonlethal damage occurs.

J. W. WILSON: The neuroblast cells you speak of are quite different from the ones in grasshoppers, are they not, because the latter do go on and divide? Your cells are really postmitotic in the sense that, in this region of the developing nervous system, there is never any further mitosis. Furthermore, the damage in the grasshopper cells is not real pycnosis, whereas yours is. True pycnotic cells never can recover so that they divide. At least that is my understanding.

The important thing about Dr. Hicks' neuroblast damage is that it occurs in less than 4 hours. It is comparable to the damage that occurs to no other cell so far as I know, except to the lymphocyte, which is also immediately damaged while not in mitosis.

HAYMAKER: I think we all know what Dr. Hicks means by "neuroblasts" in the subependymal matrix, but I do not believe that many people would understand that term if they had not heard Dr. Hicks speak. He is using that term in order to simplify, for he himself has pointed out that these cells are undifferentiated. Some of the cells are doubtless spongioblasts which have the capacity of developing into neuroglia and neuroblasts. It would seem more appropriate to refer to these cells by the noncommittal term "subependymal matrix cells," for several cell types and stages of development are concerned. A neuroblast is a specific cell en route to becoming a nerve cell. The cells involved in the superficial layer of the cortex, so convincingly illustrated by Dr. Hicks, appear to me to be well differentiated, though young, nerve cells rather than "neuroblasts."

SCHJEIDE: Dr. Hicks, you state that the metabolic activity is possibly higher in those regions of the embryonic brain which are affected by irradiation. Does this mean that the rate of cell division is also higher in these regions of the brain?

HICKS: Mitotic figures are rare in the zone of radiosensitive cells in the embryo.

SCHJEIDE: Your observation of only a few mitotic figures in fixed brain tissues does not preclude the possibility of a high mitotic rate. For example, in the hematopoietic tissue of tadpoles we see only a few mitotic cells unless we first inject the animal with a certain dose of colchicine and then wait a few hours before killing the tadpoles. In this case the accumulation of arrested mitotic figures reveals that there is indeed a rapid rate of cell division in these tissues.

HICKS: After colchicine we still see relatively few mitoses in the radiosensitive cells.

VOGEL (with lantern slides): X radiation was delivered to the heads only of 1- to 2-day-old mice (C3H × C57Bl F_1). The bodies were protected by lead shielding. Histological damage to the retina was studied at hourly intervals during the first day after irradiation and at daily intervals thereafter. Marked necrosis and pycnosis of the nuclei were observed 3–4 hours after the X irradiation. The mouse retina is largely embryonic at birth, being made up of a single undifferentiated layer. The layer of undifferentiated cells that will become the sensory rods is completely destroyed by the radiation, while little damage is seen in the ganglionic layer. Rosette formation, as in the brain, is commonly observed.

It is suggested that the retina is a useful tool in which to study the differentiation of embryonic cells (in an adult) and their relative radiosensitivity.

HICKS: In this published manuscript, but not in my oral presentation here, I discussed the disadvantages of the term "neuroblast." We all recognize that the radiosensitive neural cell is differentiating, it is in a stage beyond the most primitive neurectoderm. It is a precursor of neurons and glia, but in the radiosensitive stage, it has not yet shown, by ordinary methods, what its ultimate goal will be. The same is essentially true for the retina.

HAMBURGER: I would agree that a better term than "neuroblast" should be found. Why not "medulloblast"?

HICKS: In pathology, medulloblast has been used in several ways, just as has the term neuroblast. For the radiosensitive cell the term primitive neuroblasts and spongioblasts seems presently useful.

HAMBURGER: I think if a sufficiently large group of people agree on a term, the pathologists might be persuaded to accept it. Of course I, probably like everybody else here, was impressed in seeing what seemed to me good mitotic figures side by side with heavily damaged differentiating cells. Could it be that in a particular stage of differentiation of a nerve cell the cytoplasm is more radiosensitive than the nucleus?

RUGH: This may be overly enthusiastic, but I wish it were possible for Dr. Hollaender to have these three papers by Drs. Hicks, Wilson, and Russell abstracted, maybe not with a picture of the anencephalic baby rats on the front but abstracted and sent to every clinical radiologist in the country. The reason is that it was estimated that last year over 2,500,000 women in this country were exposed to X rays from 5 to 80 r, in clinical diagnoses; and it is anybody's guess as to how many thousands of those women were pregnant within the first 3 or 4 weeks.

Dr. Hicks has made the statement to me that a dose of as little as 40 r to the rat embryo at a time comparable to the 20-day stage in the human is extremely damaging to the central nervous system. So I think it is extremely important that the essential points of these three papers be abstracted and brought to the attention of clinical radiologists throughout the country. While mouse and rat experiments cannot be extrapolated to the human, the results are suggestive.

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EFFECTS OF RADIOMIMETIC SUBSTANCES ON EMBRYONIC DEVELOPMENT, WITH SPECIAL REFERENCE TO NITROGEN MUSTARDS

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Knowledge of the basic effects of radiation on embryonic tissues in terms of biochemical or chemical reactions that can be integrated into the biological events is rather fragmentary. Yet the list of effects caused by radiation on the cytology, histology, and the metabolic behavior of cells is a large one. Many of these types of effects are mimicked by chemical substances, but the observed similarity may be only superficial and does not necessarily indicate an identity of causation. Also it is well to remember that the number of ways in which a cell can break down is limited, but the causes of these happenings are legion. In full realization of these complications only a few substances have been selected for discussion, but these, especially the nitrogen mustard, cause cellular and developmental effects that mimic in many aspects those produced by radiation.

THE EFFECT OF NITROGEN MUSTARD ON EMBRYONIC AMPHIBIAN DEVELOPMENT

The nitrogen mustard compound, methyl-bis(β-chloroethyl)-amine hydrochloride (MBA), has pronounced effects on the development of amphibian embryos. Its action has been studied in detail on several developmental systems in this material (Bodenstein, '47, '48; Bodenstein and Abdel-Malek, '49; Bodenstein and Goldin, '48; Bodenstein and Kondritzer, '48).

When young embryonic stages of *Amblystoma punctatum* are exposed for 45 minutes to an 0.001% solution of MBA, development is partially inhibited and severe developmental disturbances are revealed. Even a superficial examination of the external effects shows that the centers of proliferation are predominantly affected. In general, it was found that the earlier in development the treatment was applied, the more pronounced was the inhibitory effect on the developing organ systems. The detailed histological effects are well illustrated in the developmental behavior of the embryonic eye. Here two distinct cellular reactions are elicited which occur in a definite temporal spatial order. Certain cells in the eye are found to break down rather soon after the embryo is exposed, while other cells enlarge greatly. Some of the cells remain unaffected and continue to differentiate. Further studies reveal that the cells comprising the germinal zone at the eye rim are the ones that enlarge and at the same time cease to multiply. The mitotic cycle is arrested at the interphase but the cells continue to grow, and reach giant proportions. The cells that break down soon after treatment without undergoing an initial enlargement are the nondividing cells of the embryonic retina. Not all these presumptive retina cells break down, but the distribution of the broken cells follows a definite spatial pattern. Exposure of increasingly older embryos to a 0.001% solution of MBA reveals that the older the eyes, the more of the retina cells resist the treatment. In the very young eye almost all of the nondividing retina cells disintegrate. The first resistant cells appear in older eyes at the center of the retina. As development proceeds this center of resistance spreads toward the eye rim. The resistance parallels the progress of differentiation of the various retinal elements. Exposure to various concentrations of MBA shows that the destruction of the eye tissues increases with the rise in the concentration of the agent. The progress of destruction follows the same spatial pattern in all concentrations, but the end point of the destruction is different. Lower concentrations of MBA destroy fewer cells than higher concentrations. The breakdown of the nuclei also increases in velocity when higher concentrations are used. It is noteworthy that the defects produced in eyes of the same age exposed to different concentrations resemble those defects found in eyes treated at different ages but with the same concentrations. These results show clearly the selective action of the nitrogen mustard.

In the developmental system of the eye the centers of proliferation and differentiation are spatially separated. If the observed reactions are not limited to the eye but are characteristic of all developmental systems in this material, then other organ systems in which proliferating and differentiating cells are set apart should be affected in a similar manner. This proved to be the case. The neural tube and the spinal ganglion, for instance, are such systems. Here again it is found that, under the influence of MBA treatment, all mitotic activity ceases. And again only those cells that belong to the proliferation zone show the enlargement reaction. The cells of the nondividing organ regions either break down without initial enlargement, or in older stages, are unaffected and continue to differentiate normally. The agent which by the cell enlargement reaction makes visible the centers of proliferation thus provides a useful tool for the study of growth patterns. Moreover, the continued differentiation of the resisting cells in the absence of any mitotic activity allows control, experimentally, of the size of an organ. This may be of importance for investigating the mutual interaction of developing organs, for their relation plays an important role in the determination of organ size.

In many developmental systems proliferation and differentiation take place in the same cell. The cells of the ectoderm, for instance, develop in this manner. The results discussed have emphasized that MBA affects selectively the processes of cell multiplication and differentiation. Since the ectoderm cell is able to fulfill both functions, cessation of all mitotic activity but continued normal growth and differentiation in the ectoderm cells may be expected. This is exactly what happens. Especially striking is the effect on the presumptive gland cells of the ectoderm that differentiate under mustard treatment to giant Leydig cells of apparently normal function. The same phenomenon can be observed in other cell types that have the same mode of development as the ectoderm cells. For example, exposed cells in the region of the duodenum differentiate into giant goblet cells and the cells of the pronephros attain a giant size. There are other instances with like implication.

To summarize the main results: Under the effect of MBA, all mitotic activity ceases. The cells of the germinal zones grow to giant proportions. Other cells, including those of the ectoderm and the gut in which multiplication and differentiation normally occur in the same cell also become giant, and differentiate to functional completion. Nonmitotic cells, which from early developmental stages are set aside for differentiation, either break down completely or are not affected; their resistance to the agent increases as their state of differentiation progresses.

Certain other attributes of cellular behavior are also not affected by mustard. The migration of the presumptive pigment cells from the neural crest to the flank of the embryo is unimpeded. Similarly, the lateral line primordia retain their power of migration, although their mitotic activity, caused by the mustard treatment, has completely ceased. The early aggregation of the blastema cells in the regenerating tail of *Amblystoma* larvae proceeds normally, but since the blastema are unable to enter the proliferation phase, regeneration fails.

EXPERIMENTS ON THE DEVELOPMENTAL BEHAVIOR OF INSECTS TREATED WITH MUSTARD

In principle, the results of mustard treatment on developing insects are similar to those on amphibians, although the manifestation of certain effects may be modified in insects in accordance with their specialized developmental behavior. The relative insensitivity of the differentiating or differentiated cells toward MBA is well illustrated by experiments on *Drosophila* larvae. Exposed to an appropriate amount of

MBA, the young larvae usually continue their larval development, but once fully grown they fail to pupate. A close examination of such individuals reveals that their imaginal discs have remained undeveloped. The discs are present but are about as small as they were at the time the animal was exposed. These small discs are apparently too feeble to respond to the pupation factors and the larvae are, therefore, unable to pupate.

From this it is obvious that the discs, being the germinal regions of the imaginal structures, are the loci most severely affected by MBA. Another example with like implication is provided by a study of the brain of mature *Drosophila* larvae after treatment at younger stages. The brain hemisphere of the last stage normal larva contains within its tissues the anlagen of the future eye ganglia in the form of a rather complex, hollow, cup-shaped center of cellular aggregation. The anlagen of these eye ganglia are histologically very prominent in last stage individuals, while they appear much less conspicuous in younger stages. In the brain of treated mature larvae the ganglion anlagen are either very small or absent. This gives the treated brain an entirely different appearance from that of the normal brain of this advanced stage. Cases in which the ganglion anlagen are missing and the organ discs are much reduced in size, but in which the purely larval structures are apparently unaffected, supply ample evidence for the predominant effect the mustard exerts on proliferating regions, and, at the same time, show that the differentiated larval tissues are quite insensitive to MBA.

Less severe exposures to MBA produced in *Drosophila* interesting modifications of the principles related. The induction by MBA of a developmental modification known as aristapedia is a good illustration of this (Bodenstein and Abdel-Malek, '49). In aristapedia, the arista of the insect is transformed into a leg-like structure. The aristapedia character occurs after exposure to MBA at a definite larval stage. Thus there exists a mustard-sensitive period for this effect. Recalling the specific inhibitory action of the nitrogen mustards on cell division in the imaginal discs discussed above, the explanation for the aristapedia phenomenon must be sought for here. Evidently, the proliferation of the anlage that gives rise to the aristapedia could not have been suppressed totally, for the antenna to which the foot-like structure is attached, as well as the modified arista itself, are of considerable size. The imaginal disc must, therefore, have continued to grow after exposure. But mitosis in certain sensitive regions of the disc, having been arrested for a limited time, might well have altered the growth pattern of the disc. The situation encountered here may be similar to that observed in the amphibian eye, where specific regions showed a special sensitivity to MBA. If an alteration in the growth pattern could account for the transformation of presumptive arista material into leg material, a regional inhibition of proliferation could well explain the phenomena observed. We believe that this is possible (Bodenstein and Abdel-Malek, '49). This assumption gains support from the observation that other mitosis-affecting agents, such as ultraviolet radiation, X ray, ether, and colchicine, bring about developmental modifications of similar types; and from the actual production of aristapedia by X rays (Waddington, '42) and colchicine (Vogt, '47). The importance of these experiments lies in the vital part played by the growth patterns in determining the characteristics of embryonic differentiation. It is conceived (Bodenstein and Abdel-Malek, '49) that the general principle involved here concerns, essentially, a competition of developmental fields for lead.

The state of differentiation of the bristle-forming cells in *Drosophila* is reflected in their selective sensitivity to mustard. For example, after exposure of mature larvae to MBA the bristles on the head of the adult fly may be completely missing. Exposure of young pupal stages removes, in addition to the head bristles, the bristles from the thorax of the fly. A somewhat later exposure has no effect on the head and thorax bristles, but prohibits formation of the dorsal abdominal bristles. Exposure as late as 2 days after puparium formation does not further affect the dorsal abdominal bristles, but now the bristles on the ventral surface of the abdomen are absent. Later treatment has no effect on bristle formation. Although this regional sensitivity associated with age is clearly demonstrated, it is found that not all bristles within a given area are equally sensitive. Certain bristles in an early responsive anterior region may, for example, persist until the major effect has shifted to the next more posteriorly located region. Thus there exists a definite order of resistance to MBA within the bristle pattern, the details of which have not yet been worked out. Yet, even with these deviations, the fact remains that the increase with age in the resistance of the bristle-forming cells to mustard progresses in an anterior-posterior direction.

Now it is noteworthy that the visible differentiation of the bristles, i.e., bristle pigmentation in later stages of development, proceeds in the same regional sequence. The bristles appear first on the head on the pupa, then on the thorax, then on the dorsum of the abdomen, and finally on the ventral side of the abdomen. Since the early pigmentation of the head bristles may be taken as an indication of their advanced state of differentiation and since these bristles are the first to become resistant to MBA, it appears that the state of bristle differentiation is causally related to the state of their resistance. This implies that the phenomenon witnessed here is the same as that already observed in the case of the differentiating retina cells in amphibians.

THE SITE OF ACTION OF NITROGEN MUSTARD IN EMBRYONIC CELLS

The amphibian experiments will provide information as to the site of action of MBA (Bodenstein, '47; Bodenstein and Kondritzer, '48). Here those cells in which growth and differentiation continue after exposure to mustard provide the first pertinent facts for the elucidation of this problem. The cells of the treated ectoderm will serve as an example. It should be recalled that these cells lose their ability to multiply. A closer study of this phenomenon reveals that the mitotic cycle is arrested at the interphase. After exposure, mitosis continues almost normally to the interphase, at which stage the affected cells remain. But these arrested interphase nuclei continue to grow, and reach giant proportions. Step by step with the increase in size of the nuclei, the entire cell enlarges and continues to differentiate. The presumptive gland cells of the ectoderm, for instance, differentiate into so-called Leydig cells which possess, except for their giant size, all the characteristic features of normal functional cells of this type. This implies that the cytoplasmic differentiation of the cell is unaffected but that the mustard has selectively affected the nucleus. It is significant that the mitotic events proceed unimpeded to the interphase and that nuclear growth continues. Thus the interphase activities are not merely arrested. The effect is more specific, for the nucleus retains the power of growth which is so characteristic for it at this stage. Apparently, the agent interferes specifically with the mechanism which, in the normal course of events, is responsible for the cessation of the growth period and restoration of the active mitotic state of the cell. This looks very much as though the nucleus has merely been rendered endomitotic — a well-known condition in which a polyploid chromosome number is attained without nuclear division. However, this is unlikely because normal interphase ectoderm nuclei have two nucleoli. There are also two nucleoli, although of giant size, in the exposed ectoderm cells. Since the number of nucleoli in the ectoderm of amphibian cells corresponds to the number of chromosome sets present (Fankhauser, '45), the diploid condition of the exposed cells is fairly certain. Evidently, no multiplication of chromosomes takes place in the growing nucleus. This observation was taken as an indication that the mustard had interfered with the mechanism of deoxyribonucleic acid (DNA) synthesis. On the other hand, the large nucleoli of the exposed cells and their highly basophilic condition suggested an abundant synthesis of ribonucleic acid (RNA). To test this assumption, the DNA and RNA content of exposed and normal animals was analyzed chemically (Bodenstein and Kondritzer, '48). These determinations revealed that the synthesis of the RNA was not affected by the treatment, but that the synthesis of the DNA was inhibited.

It is hardly necessary to stress that specific effects of mustard on the nucleic acid metabolism have also been observed in other biological material, and these effects were in many instances similar to those of X ray. But since these findings do not directly involve embryonic cells, they are beyond the scope of this presentation. There is, however, one other aspect of the problem that invites short comment. Do we know anything about the mechanism which causes the sudden breakdown of the retina cells? Obviously, the reaction exhibited by these cells under the influence of MBA is quite different from that shown by the ectoderm cells. It will be remembered that the breakdown reaction of the retina cells involves predominantly nondividing nuclei. These nuclei differ in their sensitivity to MBA according to their location in the retina and to their age. The breakdown of the nuclei can be hastened, delayed, or prevented by varying the concentration of the agent. Yet, in spite of this, the mode of the breakdown once it occurs varies little. There are certain indications (Bodenstein, '48) that differing sensitivity of these cells to mustard may be explained by assuming a difference in their permeability. It would be of interest to combine mustard and permeability experiments in order to alter the resistance of these cells and thus protect them from the mustard. Such investigations may also provide a way of finding a method through which the action of mustard might be directed toward specific cell types.

The reason for the nuclei breaking down in the typical manner they do must still be explained. Although this breakdown reaction, as stated above, takes place predominantly in nondividing cells, it may also involve some dividing nuclei of temporarily germinal cells. In the latter case and in contrast to the ectoderm cells, the dividing retina nuclei do not continue their division toward the interphase. They break down in any mitotic stage provided the particular cell involved is a sensitive one. If the mustard affects DNA metabolism of the retina cells as it does the cells of the ectoderm, then evidently the manifestation of its effect in the retina cells is quite different from that observed in the cells of the ectoderm, indicating that in the case of the retina a different process in the DNA metabolism is involved. This is perhaps not astonishing if it is realized that the agent is an actively dividing or resting nucleus, quite another metabolic condition from that prevailing in the interphase nucleus. The affected system is so vital for the maintenance of the embryonic retina nucleus that its destruction causes the sudden breakdown of the latter.

Now it has been found (Gjessing and Chanutin, '46; Butler *et al.*, '50) that nitrogen mustard can depolymerize DNA *in vitro*. We do not know whether this also occurs in the living cell. Yet it is not unreasonable to ask whether the rapid breakdown of the retina nuclei may not be caused by such a mechanism of MBA action. There is a way open to test this possibility cytochemically. Pollister and Leuchtenberger ('49) have described a method that used a basic dye, methyl green, in such a manner that its staining capacity is not only specific for DNA but also for the physical state of the nucleic acid. Methyl green stains polymerized DNA and is as specific as Feulgen, but in contrast with Feulgen staining, DNA in a depolymerized state is not stained by methyl green. Evidently this method is made for the elucidation of this particular problem. Accordingly, alternating serial sections of mustard-treated eye tissues were stained with Feulgen and with methyl green. The fragments of the broken nuclei gave a strong positive Feulgen reaction, but they also colored deeply with methyl green. The intensity of the green stain in the fragments did not differ significantly from that of normal nuclei. From this it certainly looks as if the characteristic breakdown of the retina nuclei is not caused by a depolymerization of DNA, but this may not necessarily be the real state of affairs. True, the fragments stain green and thus reveal the polymerized condition of the DNA content; but it is equally true that if only a small amount, for instance 10%, of the DNA had been depolymerized this could not have been detected by this method. If this stain had colored selectively the depolymerized DNA, instead of not staining it, the results would have been decisive. As the matter stands now, the question under investigation is still open.

In closing this part of the discussion, it must be stated emphatically that it is by no means established that nitrogen mustards act only on the nucleus of embryonic cells, although throughout the narrative this impression might have been given. The ability of cytoplasmic differentiation to proceed normally in the presence of nuclear defects is deceiving but does not necessarily indicate a limitation of the mustard's action to the nucleus. It is certainly possible that the mustard may have severely injured cytoplasmic components not involved in the cytoplasmic differentiation but vital for the nuclear functions. The observed nuclear effects may, at least in part, be caused by mustard-induced deficiencies in the cytoplasmic components on which normal nuclear function depends.

THE EFFECT OF SEX HORMONES ON EMBRYONIC DEVELOPMENT

Töndury and his school have revealed in an impressive series of papers (see Töndury and Cagianut, '51; Töndury, '52) their studies on the effects of sex hormones on embryonic amphibian development. Of special interest in this connection are the results obtained with the female sex hormones, estradiol and stilbestrol, used in concentrations ranging from 1:100,000 to 1:1,000,000. On the cellular level the substances cause severe mitotic disturbances and arrest the mitotic cycle in the early metaphase. Various types of mitotic abnormalities are found, all of which hinder severely a normal progress of mitosis. Many of these mitotic defects mimic those induced by X ray, ultraviolet irradiation, or treatment with other antimitotic poisons. The arrested nuclei soon become pycnotic, and the entire cell finally disintegrates completely.

Because of the selective action of these hormones on mitotic cells, the organ damage produced is greatest in regions of extensive proliferation. It is interesting that the damage inflicted on the embryonic nerve tube or the retina resembles to a remarkable degree that induced in these organs by mustard. Yet, as further analysis reveals, the similarity is only superficial.

Again the embryonic eye may serve as an illustration (Schenk, '49-'51). Under the influence of the hormone all mitotic activity ceases. Mitosis is arrested at the metaphase. Since the degeneration of the affected nuclei takes place rather slowly, there is at first an accumulation of metaphases. Because mitosis occurs at the stage of exposure not only in the permanent growth zone of the eye but also in the outer layer of the retina, the metaphase accumulation is seen at both these sites. The nuclear degeneration which soon follows leaves the affected regions filled with cell debris. Between these areas of destruction are found, however, unaffected cell islands that continue to differentiate unhindered. These resisting cells, belonging to the central eye regions which at the time of exposure had already lost their ability to divide, are apparently at a stage of early differentiation. But these are the same cells that also resisted the mustard treatment. It is for this reason that the histological pictures of a hormone- or of a mustard-treated eye appear so much alike. As far as the difference in the action of the two agents on the dividing cells is concerned, the situation is briefly this: (1) The hormone, in contrast to the mustard, makes no distinction between the cells of the permanent and those of the temporary growth zone; (2) in contrast to mustard, none of the affected cells enlarge after hormone treatment; and (3) the mitotic cycle is arrested by the hormone in the early metaphase and not, as in the mustard experiments, in the interphase.

Confronted with the problem of determining the underlying mechanism that causes the specific hormone effects, Töndury and his coworkers, led by certain considerations, focused their attention on the nucleic acids. These they studied in normal and exposed embryos by histochemical techniques. These investigations revealed that the hormones, unlike the mustards, have a strong effect on RNA. In normal resting cells the RNA is found in the form of fine, deeply staining, threadlike structures which are located around the nucleus, between the yolk platelets and on the cell borders. In the dividing nucleus these threads are less conspicuous but the spindle is now clearly stained. Under the impact of hormone treatment this characteristic picture becomes considerably altered (Töndury and Cagianut, '51). The staining capacities of the cells lessen and become irregular. The stain on the cell border is partially obliterated and so is the red zone around the nucleus. As a matter of fact, almost the entire pyronin-positive material is contracted into one lump at one point of the cytoplasm. In the exposed dividing cell the spindle loses much of its stainability, while the spindle fibers are irregularly distributed and often fragmented. It is significant that the severity of the morphogenetic organ defects caused by hormone treatment parallel in severity those observed in the distribution and content of the RNA in the affected organ regions.

This situation is especially well illustrated in the same case of the embryonic amphibian eye development (Rickenbacher, '51). In studies of the distribution and content of RNA in the normal developing eye, by means of the pyronin reaction, it was found that the cells of the eye vesicle stain very lightly with pyronin. With the formation of the eye cup and the simultaneous occurrence of extensive proliferation, the pyronin reaction increases. The onset of histogenesis in the central part of the retina is followed by a weakening of the reaction in this region. As development proceeds, larger and larger areas of the retina become pyronin negative until finally the positive reaction is limited to the cells of the proliferation zone at the eye rim. In hormone-treated eyes the over-all pyronin reaction is much weaker. The stained cytoplasmic granula, though still visible, are often clumped together. The division spindles, if stained at all, are irregular and show fragmentation. Again, the greater the number of cells in mitosis at the time of exposure, the more pronounced the disturbances.

It is interesting to note (Cagianut, '49) that the cells can be partially or totally protected from the hormone action by adding RNA to the medium. This implies that the hormone directly affects the RNA metabolism. If it is assumed that RNA formed in the cytoplasm is, in the normal course of events, needed for the buildup of DNA, then the observed chromosome defects, indicative of disturbances in the DNA metabolism, are secondary effects brought about by deficiencies in the RNA metabolism. But it must be pointed out that the assumed transformation of RNA into DNA is by no means an established fact.

Thus again an example of how different basic effects may have similar results is given by comparing the action of mustard and hormones. This shows how much care must be used in labeling a certain effect as radiomimetic, just because, in its final outcome, it resembles radiation. For, as stated in the introduction, the number of ways in which a cells can break down is limited but the causes of these happenings are legion.

INTRODUCTION TO DISCUSSION

Chairman Weiss: A most impressive feature of discussions of the first day of this meeting was a clear demonstration of the empiricism that still prevails in this whole field; and that is good. We do not have the facts with which to build big theories in this field; and, obviously, no generalizations that could have been attempted — and none of them were — would have stood up under the fire of the successive speakers and their facts.

In this state of affairs, where we do not know just what radiation does, it is well to examine a little more closely the test object being used to assay those very precise physical agents in which we are interested. This much less precise object is embryonic development, which we usually break down into such categories as growth, differentiation, and morphogenesis. These individual categories are being used as if they in turn were unitary events — which they are not — instead of highly complex affairs. The embryo is built like an automobile, on an assembly line by innumerable processes succeeding each other — some with a latitude for repair, others not. At any one point along that complicated line, some agent which we apply may strike. Sometimes it strikes right at the time we apply it and sometimes later, because of a latency of its actions. Most frequently it strikes at a time when we cannot detect it since, with our still very inadequate means of detection, that subtle change which we have produced in development does not present itself to us visibly until it has become greatly magnified by the subsequent chain of events. We do not have available for use in the embryonic system a clear-cut indicator instrument, such as the physicist has in a thermometer or barometer, that will give a direct quantitative correlation between the agent and its own excursion.

It is not only presently impossible, therefore, to say where radiation strikes, but it is even premature to assume that there must be a single common denominator to all these observed effects. There may be one, and we hope there will be, but I do not think it is yet time to set this up as a postulate. We should now keep in mind that radiation may at one time hit the process of protoplasmic synthesis, at another time interfere with the segregation of chromosomes, and at a third time strike the cell in a state in which it is not even synthesizing — in the sense of growth — or dividing at all, and have entirely different actions which register later as a malformation or a physiological defect.

In this state of uncertainty, it is highly useful to consider other agents which would produce similar changes. Experimental embryologists have long followed that course. Perhaps the first to use it systematically was Herbst in the early part of this century, when he applied deliberately some chemical agents to produce embryonic deformations. Child and his school used it in essentially the same sense with certain theoretical implications, some of which have been well borne out by modern work, while others have been refined and reinterpreted, particularly by the Swedish school under Runnström. A third approach, the application of metabolic inhibitors, has recently become very active and promises substantial advances. Some other inhibitor agents and some other similar effects will be discussed at this conference. We should listen to these things with an open mind, not assuming that they must necessarily ever boil down to a single cause or common denominator. There are many ways of development; there are many links in the process. And the various agents used may act on quite different parts of the chain.

To give you a simile, it is like trying to learn about the running of an automobile by stalling it, blocking it, or taking out some part. If we find that water will stall a motor, this does not tell us yet whether it does it by causing a vapor lock in the fuel line or by short-circuiting the electricity as a damp film on the surface.

DISCUSSION

BRUNST: Dr. Bodenstein, I thing this similarity in effects of mustard and X radiation is very interesting. Especially interesting to me was your beautiful picture of formation of giant cells, because the formation of giant cells is very typical of the effect of X irradiation, and has been described by many authors in various animals and plants.

A general picture of eye damage in your work is also similar to the damage after X irradiation. While I am not able to see the rod and cone cells in your picture, I do believe they exist. Some of your pictures show that eye pigment cells are very resistant to mustard. The same was observed after X irradiation, but there is also some difference. I understood that after some time you observed recovery of the eye from the mustard damage and a kind of regeneration of the eye.

BODENSTEIN: After mustard treatment with the doses used in my experiments, there is no regeneration of the eye. You will recall that the agent has caused a cessation of all proliferation and from this there is no recovery. However, after the broken cells are absorbed, the resisting cells try to regulate the disturbed organization of the retina. According to the amount of retinal material left, smaller or larger eyes of quite normal appearance are formed. These eyes continue their differentiation but do not grow.

BRUNST: Here is a difference because the effect of X radiation is permanent, if the dose was large enough. For example, I have observed complete suppression of regeneration even 5 years after irradiation.

VOGEL: In the early sections of the eye, was the lens actually damaged or was the damage histological?

BODENSTEIN: The developing lens is definitely affected by mustard. Although I have not studied this system carefully, I have observed cessation of mitotic activity, the formation of giant cells, and the presence of some broken cells in the lens. If I remember correctly, younger lenses seem to become opaque after treatment. Part of the opaqueness may be caused by the debris of cell fragments.

GAULDEN: Dr. R. C. von Borstel has found that nitrogen mustard treatment of *Habrobracon* eggs (unfertilized) at metaphase I limits the number of cleavages to six or seven. Cessation of mitosis in the cleavage nuclei occurs at interphase and the nuclei swell to about 200 times their normal volume. Dr. A. R. Whiting has obtained similar results with fertilized eggs by treating sperm only. Recent histochemical studies by Dr. von Borstel indicate that it is the protein and not the DNA content which is greatly increased in these enlarged nuclei.

BODENSTEIN: There is no doubt that the RNA content is increased in the giant amphibian nuclei. Whether or not the protein content is also increased I do not know, but I would suspect so. The DNA content is not increased.

GAULDEN: Do you have any idea what causes cells to break down following nitrogen mustard treatment?

BODENSTEIN: I had thought that the physical state of the nucleic acid in the nondividing nuclei might perhaps be responsible for their breakdown. Since it was known that mustard can depolymerize DNA *in vitro,* I thought that the same might occur *in vivo.* Now, Pollister and Leuchtenberger have shown that methyl green will stain polymerized DNA, but will not stain depolymerized DNA. Accordingly, I have treated the fragments of broken nuclei with methyl green and found them easily stained by this dye. Thus it appears that the characteristic breakdown of these nuclei is not caused by a depolymerization of DNA. Yet if only a small amount of DNA is depolymerized, but enough to cause the nuclei to break down, this cannot be detected by the method used. Therefore this question is still not settled.

WEISS: Does not the mammalian red blood cell maintain itself very well without any depolymerized DNA? Since it has no nucleus and no DNA, I do not think it can make any difference whether the absent DNA is polymerized or depolymerized.

BODENSTEIN: Although red blood cells have no DNA, I believe that it does not necessarily follow that other cells which contain DNA can get along without it.

HERMANN: The methyl green stain will polymerize DNA. Dr. Koernig has recently shown that the binding of methyl green by DNA is the same for a long period of time when you start depolymerizing DNA, and only after viscosity has almost completely disappeared does the methyl green binding drop off. So I do not think it is a valid test.

BODENSTEIN: I realize this now, but at the time I made the tests this was not known.

BRUNST: I observed an extremely large size of giant cells also in the mouse tissue after the whole-body irradiation, 10 months after treatment.

WAELSCH: Is there any chance that the increase in cell size is due to swelling? Are you sure it is due to an increase in protein content, and how was that measured? Also are your measurements of DNA done with microchemical or photometric methods?

Kemp: It was noted that those eyes treated with nitrogen mustard in the younger stages were smaller than normal at later stages, and also that the vitreous chamber was lacking. The lens was pressed against the retina. I have been able to simulate the same condition merely by excising the heart in larvae either of *Rana pipiens* or *Amblystoma punctatum* after circulation has been established.

Within the first day after this operation, the vitreous chamber collapses, indicating that the hydrostatic pressure maintained by circulating blood in the eye accounts for that feature of its morphology. Also, even one day after excision of the heart sometimes there was pycnosis of the central nuclei of the retina. I would be willing to predict that, in your experiments, undoubtedly one of the effects of nitrogen mustard is to cut off the circulation. In the ones that were treated at older stages, the eyes remained more normal, retaining the vitreous chambers. It is logical to infer that in those the circulation was not knocked out.

Bodenstein: The eyes of embryos treated at older stages appear more normal because the retinal cells are more resistant to mustard. Mustard does affect the circulation, yet I do not think that this in turn affects the eye.

Weiss: It will be interesting to hear some comments about whether or not these substances are really acting on specific localized cell groups or whether their effects are localized merely because those are the sensitive types of cells in that particular stage, and they will be sensitive to practically any noxious agent applied at the time. Is there really something in common to the agents that produces this particular effect?

Particularly striking, of course, is the similarity to what Dr. Hicks showed us yesterday, the elimination of that one subgerminal layer of neural cells of the neonate. Dr. Hamburger, can we be absolutely sure that those cells lying right beneath the ependymal layer are not the resting phase of the germinal cells themselves, but that at the time cell division occurs and mitotic figures actually become evident, the mitotic nucleus has moved down toward the interface of the central canal? That is where we find them. But during interphase, might not the nuclei of potentially dividing cells lie farther inward, in subsurface layers?

HAMBURGER: It seems to me that the subgerminal layer must be rather heterogeneous. According to the papers of Sauer, during the period of proliferation, cells move constantly from the subgerminal layer into the germinal layer, divide there, and move back into the mantle. In addition to these proliferating cells, there must be also those cells in the subgerminal layer which are in initial stages of differentiation and on their way to the outer mantle. Therefore, it is quite possible that you have a selective action on either one of these cell types, which are not always visibly different from each other.

BODENSTEIN: Possibly the ependymal layer contains a diverse cell population. But it seems to me significant that cell divisions occur predominantly in the ependymal layer and that it is here that the nuclei show the typical enlargement reaction. One must also bear in mind that giant nuclei occur in other organs only at those regions containing potentially mitotic cells. A good example of this occurs in older eyes where the region of proliferation is rather sharply restricted to the eye rim. As far as the neural tube is concerned, I am quite willing to believe, as Dr. Hamburger has pointed out, that the ependymal layer may contain, in addition to proliferating cells, some that are in early phases of differentiation; but I consider the latter as rather exceptional. I might also point out that, occasionally, one finds a division nucleus in the region of the outer mantle, but this, too, is rare.

WEISS: In the first place, when we talk about cells we mean nuclei. I think we should keep that clearly in mind. The cells span the plate at that stage, only the position of the nuclei within the cells varies. I think that is very misleading language, although we all, including myself, are using it. But the second question is: If these cells are disintegrated, if only a fraction of them is broken up, what about a secondary action from this necrotic tissue? If you have a tissue in which parts are completely necrotic, could not there be a secondary action? Is that something to consider both for radiation and for these mustards? If you have some primary targets, so to speak — and they need not necessarily be the dividing cells but may be the ones that synthesize protoplasm, including more DNA — if they are hit, then they break up and then, as a result of this, you get a secondary action of some other kind spreading, which is beyond our control.

Bodenstein: The reactions I have discussed must be considered as direct actions of the mustard on the target. Whether the mustard acts on the nucleus only, as we were inclined to believe when this work was done, seems to be doubtful now. Recent evidence by Danielli indicates that the cytoplasm might also be involved. The observed nuclear effects may thus, at least in part, be caused by mustard-induced deficiencies in the cytoplasmic components on which the normal nuclear functions depend (see also Dr. Noonan's discussion).

It is very timely that Dr. Weiss has brought up the question of secondary effect, i.e., the possible injurious effects of necrotic tissues, etc. Such effects certainly do exist and are well illustrated in the transplantation of mustand-treated tissues to normal hosts. Under such conditions, the exposed tissues may develop further and remain alive longer. The earlier death of the host animal is apparently caused by secondary effects of the kind mentioned. These effects usually develop rather late and may be detected as such.

Chairman Weiss: As a matter of fact, you can produce them by injuring the nervous system on one side, as Harrison and Detwiler have done, and you get mitosis occurring throughout the mantle.

Butler: Years ago in studies of the irradiation of the neural tube of a developing chick embryo, I found that it was possible to choose a dosage of radiation that did not apparently affect the cells in the mantle layer and did not affect all cells in the ependymal layer. By proper governing of dosage, one can bring about an effect on those cells in the ependymal layer that are in certain phases of mitotic activity and can leave other cells apparently undamaged.

So in line with the discussion that has just been going on, I agree that we have to consider a diverse population of cells in the ependymal region and not simply speak of the ependymal layer as a uniform layer of cells.

Bodenstein: In accordance with my earlier comments, I agree with Dr. Butler that the ependymal layer may well be composed of potentially different nuclei. I might add that it is also possible, with mustard, to choose a dosage that is much less effective than the one used in the experiments reported. In such a case the cell damage is much less severe and the nuclei mainly affected are located in or near the ependymal layer.

Furth: My knowledge is secondhand. Watching developments in this field, I note that the term ''radiomimetic'' was introduced when substances were found which depressed hemopoietic tissues as do ionizing irradiations. Further research disclosed marked differences and some workers proposed to abandon the term ''radiomimetic.'' The core of this problem is to define what effects are specific to ionizing irradiations; this definition will set the criteria for the term ''radiomimetic.'' It seems to me that all cytotoxic substances which do not have specific cell affinities and spread in the body mimicry ionizing irradiations in that they are capable of destroying selectively sensitive cells. However, they differ widely with respect to the mechanism of injuring or destroying cells.

Chairman Weiss: Dr. Furth, I do not know whether you know this, but I have taken a privilege which a chairman should not take of reversing the order of words when I read the title of this paper. I have not said ''the effects of radiomimetic substances'' as it states in the announcement. I called it ''radiomimetic effects of certain substances,'' and perhaps that might be a way out of this situation. I do not believe there are radiomimetic substances. Not the substance, but its effects mimic radiations. So yours is a very pertinent question. I think the closer we stick to the facts for the time being, the better off we will be in the end.

However, as for the actual point of attack, I do not believe the experiment of Danielli is absolutely conclusive because, after all, in order for a nucleus to be affected, it must be exposed to the substance; and transplanting a healthy nucleus into a cytoplasm that has been contaminated, is simply exposing the nucleus to the contaminating substance, so that in the end, the effect may still be on the nucleus just as in the reciprocal experiment. So I do not think this experiment proves anything more than that the nucleus really is involved. But about the cytoplasm, the implications are less certain.

NOONAN: In regard to this question of nuclear damage by radiation I think attention should be called to a paper by Duryee ('49). As I recall, this investigator working with frog eggs and using cytological evidence demonstrated that when the nucleus was separated from the egg and irradiated alone there was apparently less damage than when this structure was exposed to radiation within the cytoplasm. So far as I know this is the only tissue that has been investigated in this particular manner. On the basis of this report, however, it seems to me somewhat dangerous to consider that the only effect of radiation on the nucleus is a direct one. It may well be that at least some of the changes in the nucleus are the result of "toxic substances" produced in the cytoplasm by radiation.

Chairman WEISS: Of course there is one thing on which I find little discussion in the literature. Suppose you have a radiation effect which hits the nucleus directly. Of course radiation can get through the cell. Other agents cannot necessarily get through the cell as easily, and we have very little evidence of what gets into the cell and out of it in the normal healthy cell. However, during mitosis something happens to the cell. In anaphase and early telophase, the cell has its guards down, so to speak. The cell surface is completely unstabilized and disrupted in a way, and there is evidence that certain things can penetrate the cell during that particular stage which could otherwise not enter.

So I think we should consider that, when we see a mitotically active tissue do something peculiar, the primary effect may have been on some cells of that group. Then they may have broken up, released something, and this something may have become available to the other cells again, only later during the terminal phase of another mitosis.

So we again get a sort of chain reaction or relay effect which I think worth considering because it brings in mitosis, although in an entirely different form from that normally associated with the chromosome separation. By this I mean the loosening of the cell surface, when it becomes more permeable to large molecules and perhaps particulates.

ELIZABETH GREEN: In contrast to Dr. Bodenstein's finding (after treatment with nitrogen mustards) of large nuclei which do not show an increased amount of Feulgen-positive material or more than two nucleoli, we have found in eggs and embryos of *Rana pipiens* exposed to carcinogen-protein conjugates large nuclei that are characterized by more than the normal number of nucleoli and by conspicuous amounts of Feulgen-stained substance. Here there is suggestion of an endomitotic process.

There is another piece of information that came out of work on the frog's egg that was not designed as a study of the effects of irradiation (Briggs, Green, and King, '51). With sperm exposed to 100,000 r, motility was retained and fertilization took place. The egg nucleus was removed. The eggs started cleaving at approximately the normal rate and formed partial blastulae. No dorsal lip formed, and in grafting experiments it was shown that the cells of such blastulae were not able to differentiate or to be induced to form any structures when transplanted to suitable regions of normal hosts.

Cytological examination of the arrested blastulae showed that the heavily irradiated sperm chromatin had been distributed to a large number of cells. There was a lag in the participation of the irradiated sperm nucleus in early cleavage divisions. As a result, many blastomeres were formed which contained no chromatin. In many of these there were amphiastral division figures with no Feulgen-positive material. However, once started, the irradiated chromatin was distributed to 200–300 cells or about one-third of the cells of the partial blastulae. The ''resting nuclei'' in these cells were quite normal in appearance, although some were large and others very small. There were some division figures showing what appeared to be fragmented chromosomes. No cases were seen of actual splitting and separation of halves of the individual ''chromosomes.'' However, it was clear that such heavily irradiated chromatin was still capable of increasing in amount, if not of asserting its normal genetic influence.

BODENSTEIN: The observation that irradiated sperm, though strongly affected, retains its mobility is of special interest to me. A similar phenomenon can be observed after mustard treatment. For instance, the presumptive melanophores, although unable to divide, retain their power of movement and the same is true for the movements of the lateral line placodes.

KARNOFSKY: I want to present the following experiment to Dr. Bodenstein, and wonder if he will interpret it in the light of his reported observations. We took several different types of transplantable mouse tumors in mice, all growing at approximately the same rate. These mice were treated by total-body irradiation or by nitrogen mustard, and the tumors examined at several intervals after exposure. The effects of X irradiation and nitrogen mustard were fairly similar, but several distinctive histological patterns were found in the tumors.

For example, in the mouse sarcoma 180 the cells and nuclei were enlarged, and they were about 4 to 8 times normal size. A transplantable lymphosarcoma did not show cellular enlargement, but the nuclei were fragmented, and in a few hours after treatment the tumor was markedly reduced in size. A transplantable osteogenic sarcoma did not seem to be affected histologically. These tumors were all growing at approximately the same rate, and the dose of X rays or nitrogen mustard used destroyed the viability of the tumors, as determined by failure of the tumor to grow when transplanted to susceptible mice.

Do these tumors have different growth properties or differentiation potentialities? Are the cells dying by different mechanisms?

Bodenstein: Speaking only of potentially dividing cells, I am well aware that not all cells of this type show the enlargement reaction after mustard treatment. Even in amphibian embryos where the giant cells are so prominent, one finds that the degree of enlargement depends very much on the type of cell affected. As to your question concerning the causes of cell death, I hesitate even to venture a guess. I think that the discussion has clearly brought out how little we actually know about the mechanism of mustard action and about the differences that must exist in the various cell types affected.

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DISTURBANCE OF EMBRYONIC DEVELOPMENT BY MATERNAL VITAMIN DEFICIENCIES

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SEVEN FIGURES

The topic to be discussed represents but a small sector of the field of prenatal nutrition. Even the sector circumscribed by the title of this paper has grown too large to be covered completely in this conference. It will be necessary, therefore, to limit this presentation to certain phases of the work which demonstrate the role of vitamins in the development of mammalian embryos. Instead of aiming at completeness it seemed preferable to point out some of the ramifications of our subject which reach into other fields discussed in this symposium.

Many forms of reproductive failure may be caused by maternal vitamin deficiencies: sterility, fetal death, resorption or abortion, neonatal weakness or death of the offspring are probably the most frequent results of maternal dietary deficiency; they are results of practical importance in animal as well as in human reproduction. "Malformations" were omitted from this list since they can occur at any stage of development and play a part in many forms of reproductive failure. Under adverse conditions few deformed fetuses reach the natal period and "congenital" malformations are, therefore, relatively rare manifestations of maternal dietary deficiency. They occur in exceptional cases as the result of borderline deficiencies which injure the developing embryo but permit continuation of pregnancy. Mitigation of a borderline deficiency leads, as a rule, to the birth of normal young, but further deprivation leads to embryonic death (Warkany, '47). It is difficult, therefore, to obtain congenital malformations of dietary origin. The investigator who tries to produce such malformations resembles a tightrope walker who is constantly in danger of falling to one side or the other. It is understandable that failure and disappointments are not rare in this field; yet the results are of considerable interest, since they permit an insight into embryonic development which cannot be obtained by other methods. An attempt will be made to justify this statement by the following examples.

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Reproductive failure owing to vitamin A deficiency has been studied by many investigators (Evans and Bishop, '22; Mason, '35). In the course of such studies congenital anomalies have been observed occasionally (Zilva *et al.*, '21; Moore, '39). But it was Hale ('33, '35, '37) who first furnished convincing proof that congenital malformations can be induced in pigs by maternal vitamin A deficiency. This investigator fed gilts of known stock a diet deficient in vitamin A and possibly in other nutritional factors. The offspring showed microphthalmos or anophthalmos, accessory ears, cleft palate and harelip, subcutaneous cysts, and ectopic kidneys. Hale proved the nutritional origin of these malformations by preventing them in control animals when the experimental diet was supplemented with cod liver oil or green fodder. He also proved that genetic factors could be ruled out as the cause of the malformations, since matings of abnormal sons to their mothers or of abnormal brothers to their abnormal sisters resulted in normal offspring if the females were fed an adequate diet during pregnancy. A different type of congenital blindness caused by bony constriction of the optic nerve and attributable to carotene deficiency of the mother was reported in calves by Moore and coworkers ('35). Extensive studies along these lines were made possible when we found that congenital malformations could be induced in rats by maternal vitamin A deficiency (Warkany and Schraffenberger, '46). The diet of the prospective mother must be controlled soon after weaning. By adding measured amounts of carotene to a vitamin A-free diet the females are enabled to grow and mature without storing vitamin A. The day after conception they are given a completely vitamin A-free diet. In this way young can be obtained which show a syndrome of congenital malformations. Externally, these young may appear normal but sometimes they are characterized by marked edema. The eye, which is one of the most frequently affected organs in this syndrome, shows a variety of anomalies (fig. 1 A) but in all abnormal eyes a fibrous retrolenticular membrane is found. The retina is often folded and of abnormal structure; there are many instances of coloboma and of eversion of the retina; the iris and the ciliary body are rudimentary; the cornea may be fused with the lids; and ''open eyes'' are observed in rare cases. Malformations of the urogenital tract follow next in frequency. In many animals the lower poles of the kidneys are close together and their pelves are not dilated. In more severely affected animals the lower poles of the kidneys may be fused and horseshoe kidneys are formed (fig. 1 B) (Warkany and Roth, '48). Serial sections revealed many additional anomalies of the urogenital system (Wilson and Warkany, '48). A high percentage of these young have diaphragmatic hernias, more often on the right than on the left side (fig. 1 C). The interventricular septum of the heart may remain open beyond the age of normal closure (fig. 1 D) and aortic arch anomalies of many types can be discovered in serial section (Wilson and Warkany, '49). In these experiments rats of several strains were used and the same types of malformations were induced by maternal vitamin A deficiency irrespective of the stock employed. Females of the same strains receiving adequate diet or the experimental diet supplemented by vitamin A did not have abnormal young (Warkany and Schraffenberger, '46; Wilson and Barch, '49).

Figure 2 A shows the incidence of anomalies in one representative group of abnormal young with the vitamin A-deficiency syndrome. In this experiment 53% of the young

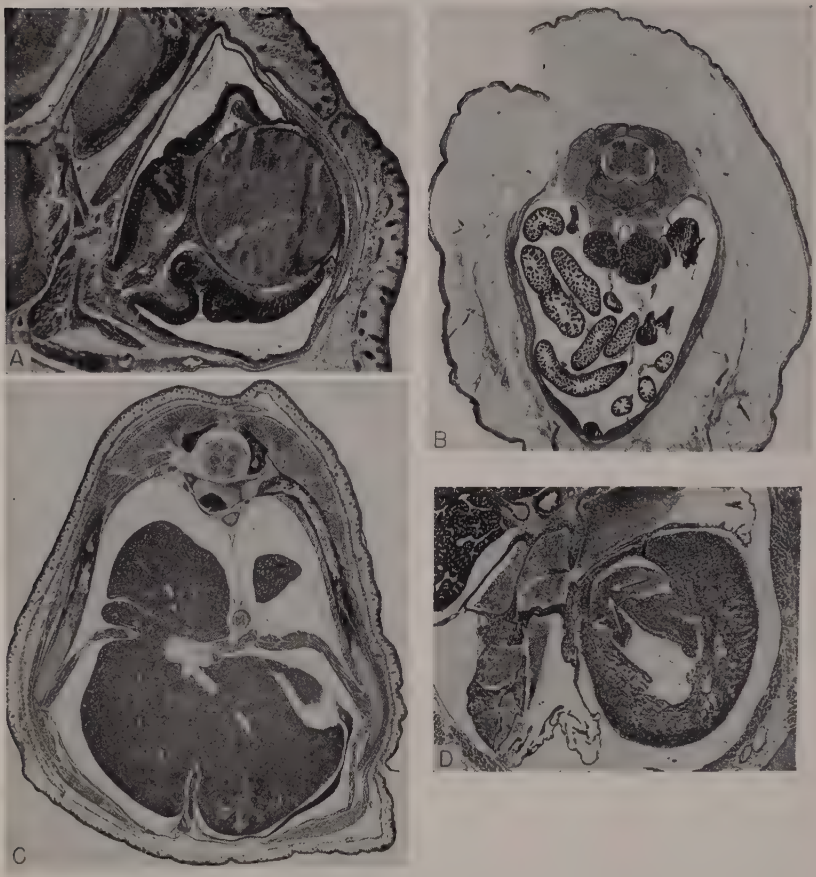

Fig. 1 Congenital anomalies in young of vitamin A-deficient female rats.

A. Section of abnormal eye (× 20) showing retrolenticular fibrous membrane in place of the vitreous body, coloboma and eversion of the retina, rudimentary iris, and ciliary body.

B. Section through abdomen (× 6) showing marked edema of subcutaneous tissue and fusion of lower poles of kidneys (horseshoe kidney).

C. Section through diaphragmatic area (× 6). The small dorsal lobe of the liver penetrates through a defect of the diaphragm into the right pleural cavity.

D. Section through heart (× 14). A blood clot (white arrows) is seen in septal defect.

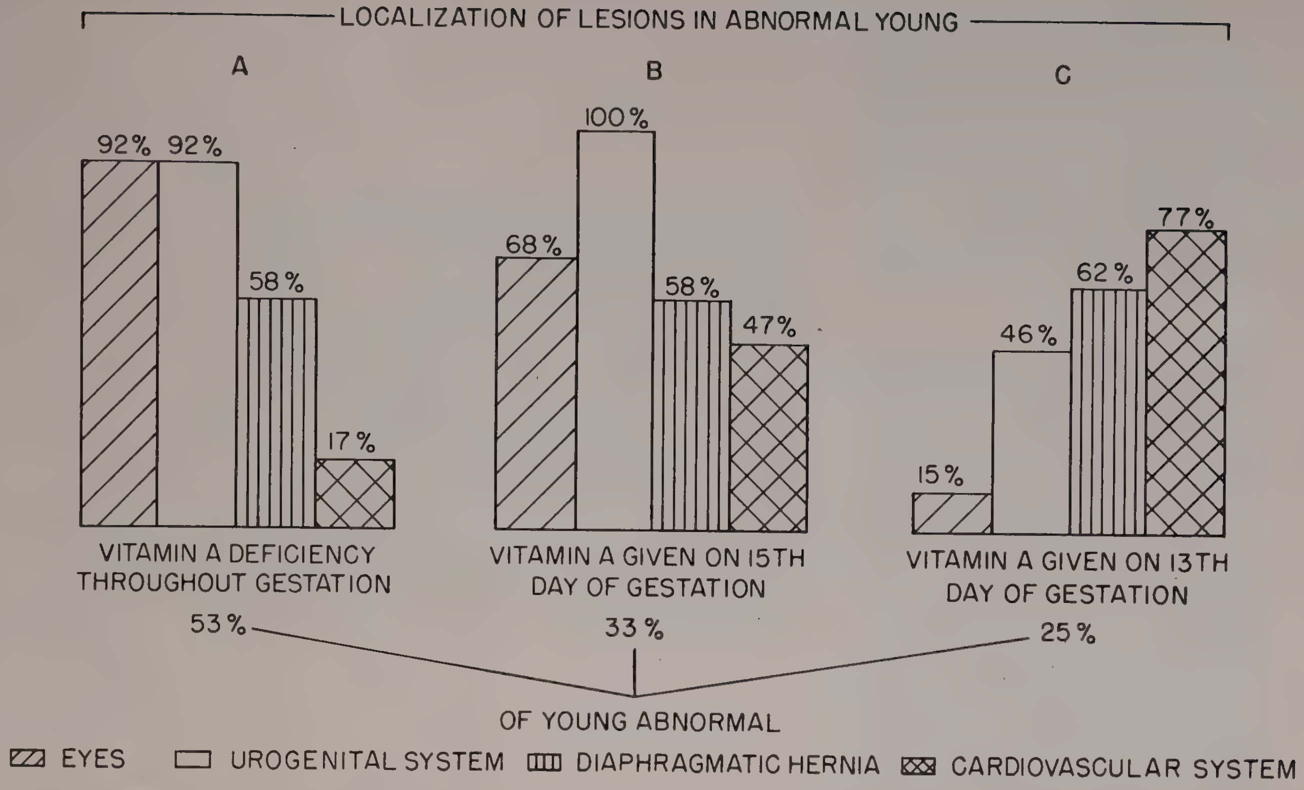

Fig. 2 Percentage incidence of affected organs in abnormal young. Vitamin A deficiency of mothers maintained: A. throughout gestation, B. through 14 days, C. through 12 days of gestation. Note change of pattern. (From Warkany, 1952–1953.)

were abnormal. Of these abnormals, over 90% had both ocular and urogenital anomalies, 58% had diaphragmatic hernia, while only 17% had cardiovascular malformations. This syndrome can be modified by interrupting the maternal dietary deficiency by administration of a single large dose of vitamin A on certain days of gestation. Vitamin A-depleted mothers were given the single vitamin A supplement on different days, some on the tenth, others on the eleventh, twelfth, thirteenth, fourteenth, and fifteenth day of gestation. It was found that the percentage of abnormal young became smaller the earlier the supplement was given. But it was also found that the prevalence of certain anomalies changed according to the date of vitamin A administration and that the pattern was affected. If, for instance, vitamin A was administered on the thirteenth day the ocular and urogenital anomalies were markedly reduced, while the incidence of cardiovascular anomalies was relatively increased (fig. 2 C). The change of the syndrome can be explained in the following way. Vitamin A deficiency damages the heart and the aortic arch system before the thirteenth day, whereas the interference with the development of the corpus vitreum of the eye occurs later. The retrolenticular fibroplasia, but not the cardiac anomalies, can be prevented, therefore, by a supplement on the thirteenth day. In figure 2 B an intermediate pattern is seen which was produced by vitamin A supplements on the fifteenth day. Thus the syndrome can be altered when vitamin A is administered during gestation (Warkany, '52–'53; Wilson *et al.,* '53).

The syndrome induced in the young of rats by maternal vitamin A deficiency consists essentially of malformations of soft tissues. A syndrome of skeletal malformations can be obtained by maternal riboflavin deficiency. Under proper experimental conditions about one third of the young born are found to be abnormal (Warkany and Nelson, '41; Warkany and Schraffenberger, '44). Many of the abnormal young can be recognized as such by external inspection. They show shortening of the mandible and of the extremities, protrusion

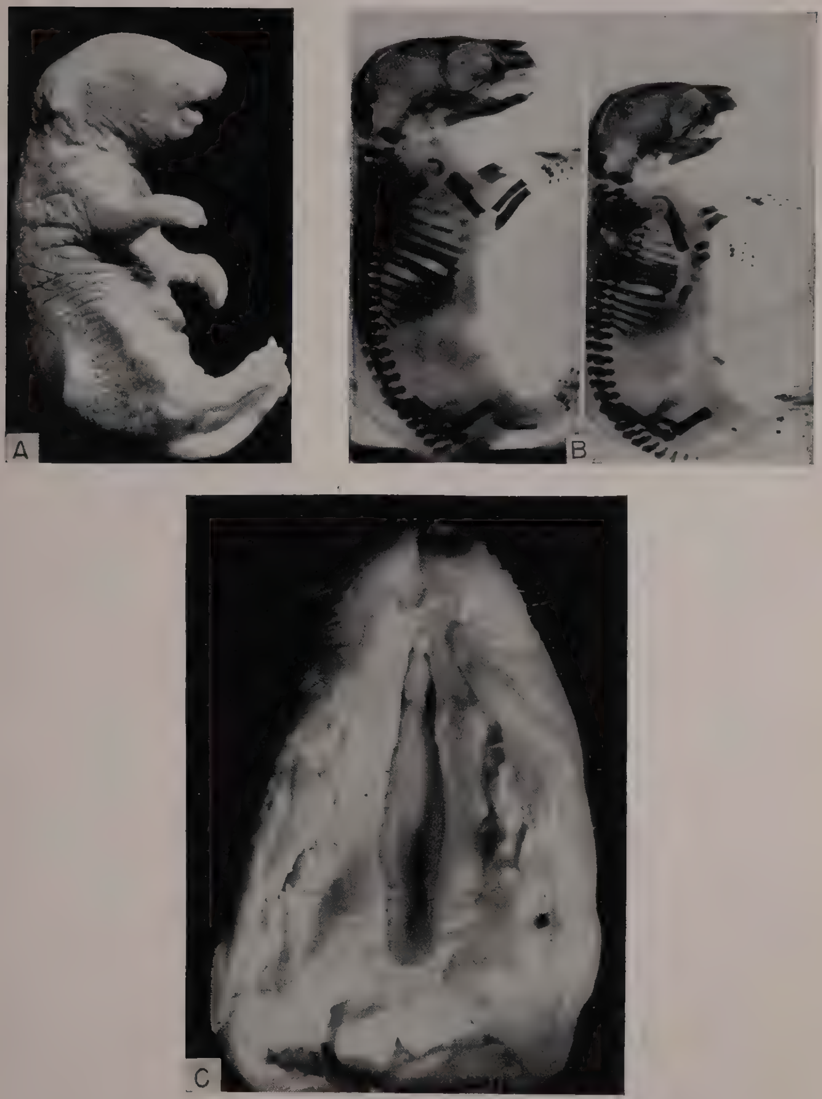

Fig. 3 Congenital anomalies in young of riboflavin-deficient female rats.

A. Young showing short mandible and protrusion of tongue.
B. Cleared specimens both showing shortening of mandible and fusion of ribs. In the right specimen there is shortening of the radius and ulna and absence of the tibia and fibula. In the left specimen the tibia appears absent and the fibula is curved.
C. Cleft palate.

of the tongue and various degrees of syndactylism (fig. 3 A) (Giroud and Boisselot, '47; Giroud *et al.,* '51; Leimbach, '49; Noback and Kupperman, '44; Piccioni and Bologna, '49). Other abnormalities are recognized after the specimens are cleared. There may be shortening or absence of the radius, ulna, tibia, and fibula. The mandible is often short and the ribs are fused (fig. 3 B). Inspection of the mouth reveals a posterior cleft palate in about one-half of the abnormal young (fig. 3 C) (Warkany *et al.,* '43b).

These young have different degrees of abnormality; but there is a pattern in this variety, in that certain bones are less often affected than others. Table 1 shows the frequency of osseous anomalies in 100 cleared abnormal specimens.

TABLE 1

Frequency of osseous defects in one hundred cleared abnormal specimens

Tibia	93	Ulna	50
Mandible	80	Humerus	34
Rib	75	Hindfoot	31
Fibula	63	Maxilla	8
Radius	58	Scapula	6
Hand	54	Clavicle	6
Sternum	52	Femur	1

Histologic sections of the abnormal bones reveal some interesting features (Warkany and Nelson, '42; Warkany *et al.,* '43a). A comparison of normal tibiae with abnormals shows retarded and irregular ossification in the specimens with malformations. In a mildly abnormal tibia the metaphyseal lines of ossification take an irregular and somewhat wavy course. Large cartilaginous areas remain enclosed within the diaphysis. The bone trabeculae radiate diagonally forward from a slightly elevated point in the center of the posterior aspect of the bones. A more abnormal tibia remains almost entirely cartilaginous although beginning ossification can be recognized in a posterior prominence of the structure. This prominence is covered by a thin shell of bone, but consists otherwise entirely of swollen cartilage cells. Anterior to these cells lies columnar cartilage, the cell columns radiating forward from the posterior bulge. A further degree of anomaly may be seen in a tibia which consists entirely of cartilage of the resting type (fig. 4).

Sections of the paws also show retarded and irregular ossification. In addition, a disorganization of the normal carpal and digital pattern may be recognized. Some bones are shortened or entirely lacking, others are wider than normal and some of the interosseous spaces and articulations are not developed (fig. 4). In some of the short tubular bones eccentric foci of ossification are seen.

It may be safely concluded from such histologic studies that the abnormal skeletal development dates back to the cartilaginous or to earlier stages of development. But it may not be learned from the sections how early in prenatal life the anomalies are determined. These studies were complemented, therefore, by experiments designed to reveal how late in the gestational period the malformations could be prevented by supplements of the deficient diet. It was found that addition of the supplement prevented all skeletal malformations of the young when the supplement was given before the thirteenth day [2] of gestation (Warkany, '52–'53). After the thirteenth day supplementation of the diet did not protect the young.

These findings suggest that riboflavin is needed for the development of the skeleton, particularly between the thirteenth and fourteenth days of embryogenesis. At this period the appendicular skeleton is still in its mesenchymal state, but its individual elements, the future bones and the joint spaces which separate them, have become recognizable. Between the thirteenth and fourteenth days of gestation the embryo is growing rapidly and is almost trebling its weight within a 24-hour period (Donaldson, '24). After this period the riboflavin deficiency does not interfere seriously with general growth and increase in weight, since malformed young

[2] In our experiments the first day of gestation is designated as the day on which sperm are found in the vaginal smear.

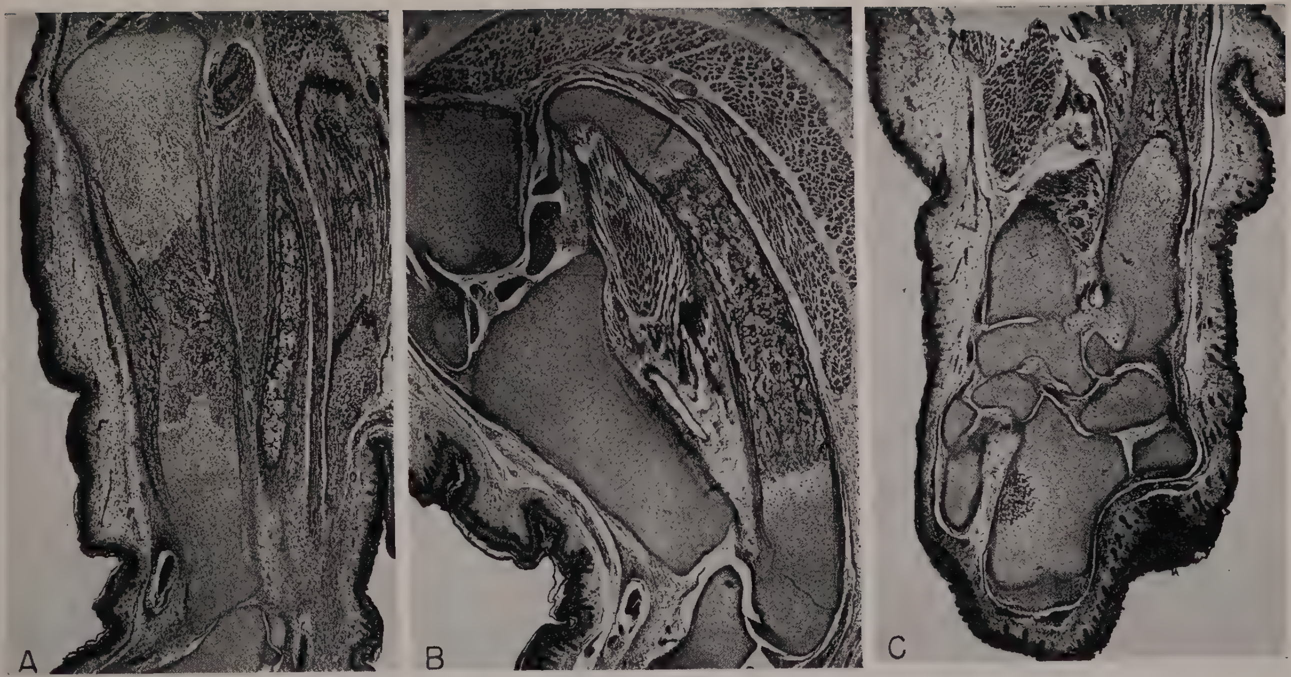

Fig. 4 Sections through skeletal structures of young of riboflavin-deficient female rats.

A. Abnormal tibia (× 16) showing retarded and abnormal ossification. Islands of cartilage are seen in the anterior aspects of the diaphysis; the osseous trabeculae take a diagonal, abnormal course.

B. Tibia short and completely cartilaginous (× 21) in the presence of a fairly well ossified fibula. Note abnormal curving of the fibula around the shortened tibia (cf. fig. 3 B left).

C. Paw (× 26). Cartilaginous bridges connect distal ends of radius and ulna with carpal cartilages. Below a broad metacarpal bone (syndactylism) is seen with eccentric center of ossification.

may be normal in size and weight at the time of birth. But the deficiency does prevent normal differentiation of the skeletal mesenchyme into cartilage, bone, and articular spaces. It seems, therefore, that at this active stage of development large amounts of riboflavin are required for normal differentiation.

To test whether our concept of this period of determination is correct we undertook in 1946 some collateral experiments using X rays as the teratogenic agent (Warkany and Schraffenberger, '47). It seems justifiable to mention here the results of that study, although they are only indirectly connected with the topic of this paper. As early as 1935 Job *et al.* ('35) employed X rays to determine critical periods in mammalian development and in 1938 Kaven ('38a, b) reported cerebral malformations after timed irradiation of pregnant mice. I wish to emphasize this fact, since some recent students (Hicks, '53) in this field have apparently overlooked the earlier work along these lines. If the time of injury to the embryo had the overwhelming importance for the morphology of malformations as the work of Stockard ('21) suggests, it should be possible to produce the same type of skeletal malformation by riboflavin deficiency and by X-irradiating between the thirteenth and fourteenth days. On superficial examination, the results of this test seemed rather gratifying, since some of the malformations produced by irradiation resembled externally those induced by riboflavin deficiency. Shortness of the lower jaw, protrusion of the tongue, cleft palate, shortness of the extremities, abduction of the hands, clubfeet, and syndactylism were malformations common to both syndromes (fig. 5). But there were many differences between the two types, the X-ray syndrome being much more varied than that induced by riboflavin deficiency. By irradiation on the thirteenth day a number of young were obtained which had encephaloceles or polydactylism, anomalies not observed in the riboflavin-deficiency syndrome. Characteristic differences were recognized after the specimens were cleared. Specimens of the two types which appeared similar

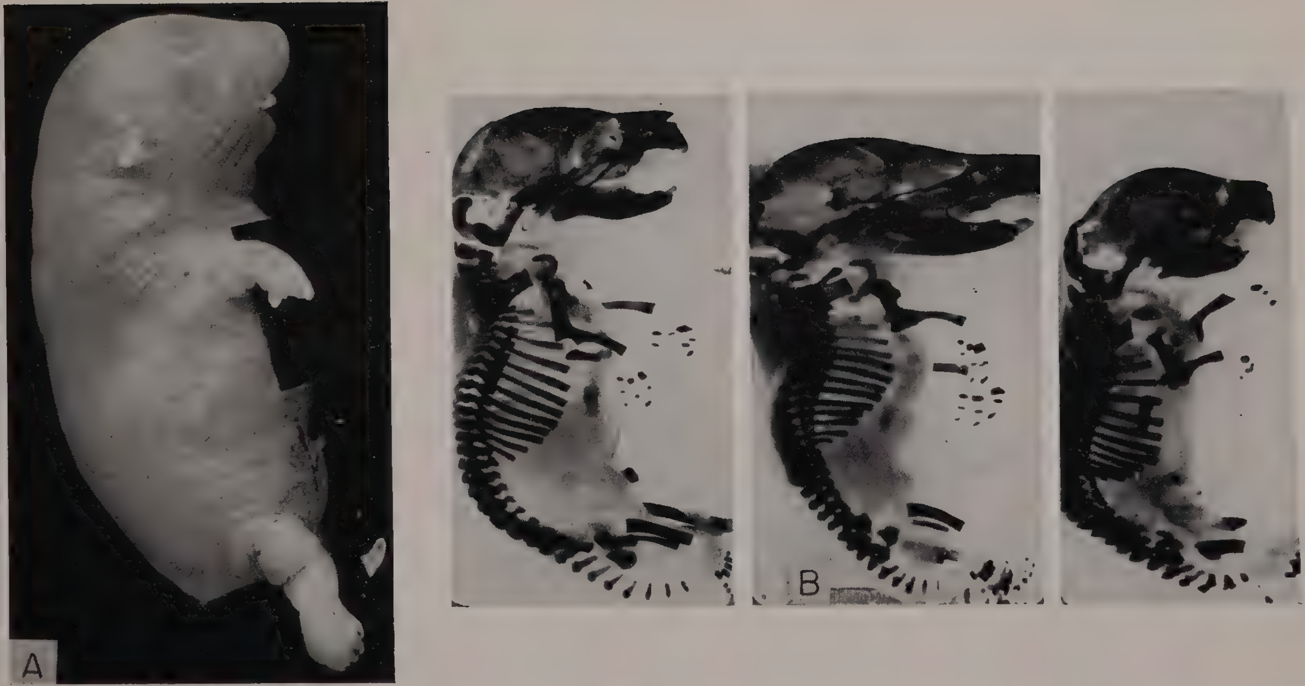

Figure 5

A. Young of female rat irradiated on thirteenth day of pregnancy (300 r). Note shortness of mandible, protruding tongue, syndactylism, and malposition of extremities.

B. Cleared specimens; young of female rats irradiated on the thirteenth day of pregnancy (250 r). Left: Shortness of mandible, humeroradial bone, shortness of ulna. Center: Humeroradial bone, absence of ulna, shortness of femur and fibula. Right: Shortness of mandible, humeroradial bone, absence of ulna and fibula.

externally could be easily distinguished by differences of the skeletal structures. In the pattern produced by irradiation on the thirteenth day both the ramus and the corpus mandibulae were reduced in size and both the ulna and the fibula were more shortened than the radius and the tibia. There was also a frequent occurrence of a humeroradial bone which was never observed in several hundred abnormal specimens obtained from riboflavin-deficient mothers. In the pattern of riboflavin deficiency, the shortening of the mandible was due to a reduction in size of the corpus mandibulae; the ramus was of normal size. The radius and the tibia were usually more shortened than the ulna and the fibula. Histologic sections revealed further differences. In the irradiation syndrome sections of an arm with osseous ankylosis of the humerus and radius showed absence of the distal epiphysis of the humerus and of the proximal epiphysis of the radius, the osseous trabeculae of the two bones being connected without demarcation (fig. 6). Sections of the brain and other internal organs (fig. 7) showed anomalies not seen in the pattern of riboflavin deficiency (Russell, '50; Wilson and Karr, '51; Wilson *et al.*, '53). Comparison of the two syndromes permits the following conclusions: It is probably correct that the riboflavin deficiency exerts its deleterious influence about the time of the thirteenth day of gestation. But, as would be expected in a chronic teratogenic experiment, the time of determination cannot be exactly defined. Another conclusion is that the time element must not be overemphasized to the neglect of other factors, since specific injurious agents influence development in specific ways. Although similar anomalies can be produced by different teratogenic factors, variances can be found on thorough examination in the anomalies as well as in the syndromes which are formed.

Returning to malformations of maternal dietary origin, it should be pointed out that vitamin B deficiencies other than that of riboflavin have been investigated, and some were found to be capable of inducing malformations. In some instances the missing dietary factor could not be established,

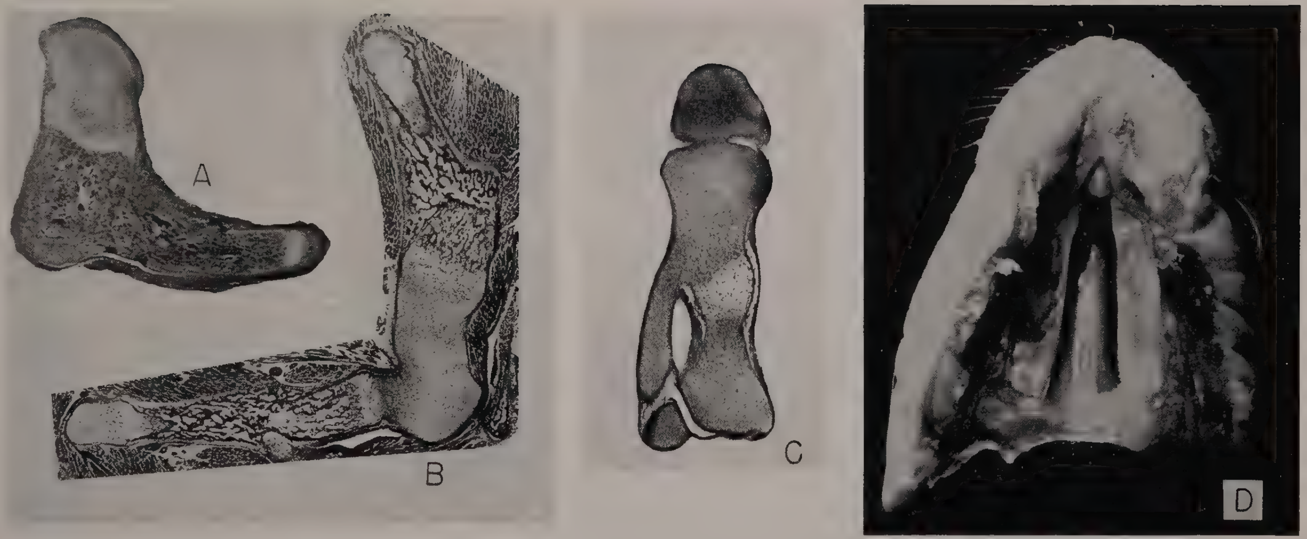

Fig. 6 A–C Sections through bones of young of mothers irradiated on the thirteenth day of pregnancy (250 r).

A. Humeroradial bone (× 16) showing firm osseous connection between humerus and radius.
B. Humeroradial bone (× 16) showing cartilaginous union of structures and lack of development of elbow joints.
C. Fibula has remained cartilaginous but beginning ossification is noticeable in the tibia. Since the proximal tibiofibular joint has not formed, the two bones appear fused.
D. Cleft palate in young after irradiation of mother on the fifteenth day of pregnancy. (Fig. 6 B, C, D from Warkany and Schraffenberger, '47.)

but it seems that the multiple malformations observed in pigs by Ross and coworkers ('44) and hydrocephaly encountered in rats by O'Dell *et al.* ('51) can be attributed to lack of factors of the vitamin B complex. Giroud and Boisselot (Giroud, '52) observed congenital malformations of the eye and of the brain which they attributed to pantothenic acid deficiency.

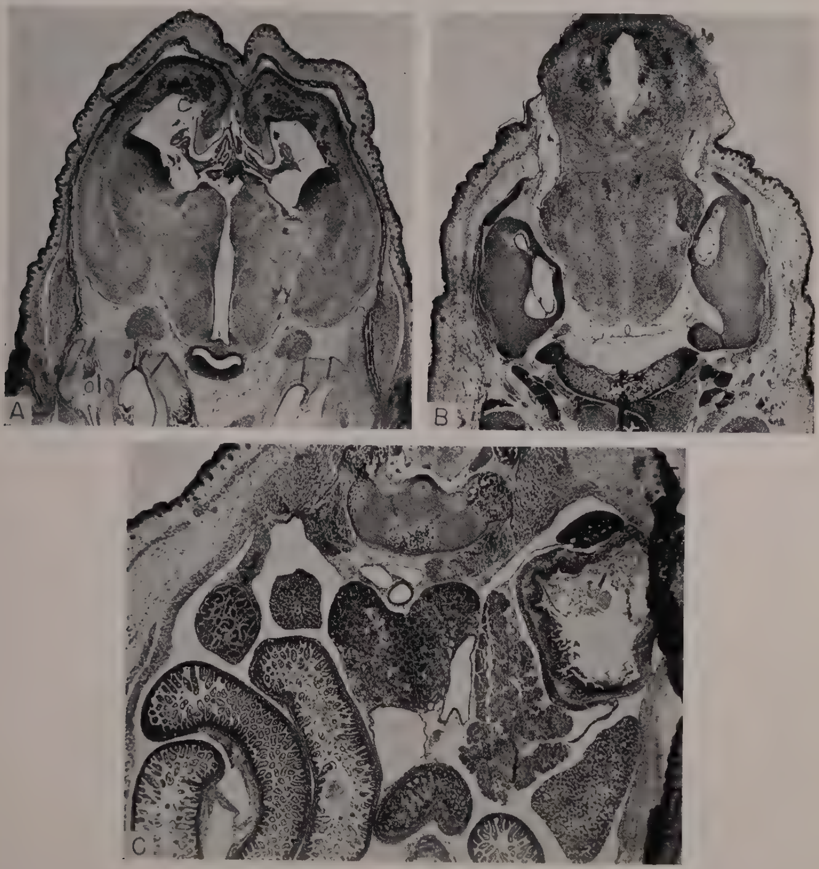

Fig. 7 Section of internal organs of young of female rats irradiated on the thirteenth day of gestation.

A. Abnormal brain (× 9) (300 r).
B. Abnormal brain protruding through skull (× 10) (780 r).
C. Median, fused kidney (× 13) (780 r).

Karnofsky and others ('49; Snell and Cravens, '50) induced congenital malformations in fowl by pteroylglutamic acid deficiency and Giroud and Lefebvres-Boisselot ('51; Giroud and Lefebvres, '51) by a similar deficiency obtained congenital abnormalities in rats. By incorporation of succinylsulfathiazole and a pteroylglutamic acid antagonist (x-methylpteroylglutamic acid) into a maternal diet lacking pteroylglutamic acid Nelson, Asling, and Evans ('50, '52) have recently produced multiple congenital abnormalities with regularity in the young of rats. Thus, by combination of a dietary deprivation with the feeding of an inhibitor and of an antagonist, an effective pteroylglutamic acid deficiency was produced. In this interesting investigation it was found that complete resorption of the embryos invariably occurred when rats were placed on the diet the day of breeding (Nelson and Evans, '47). If, however, the deficient diet was instituted on the tenth day of gestation,[3] 100% of the young born were deformed. When instituted on the eleventh, twelfth, and thirteenth days 95, 65, and 30% respectively were abnormal; when the mothers were submitted to the deficient diet as late as the fifteenth day none of the young were abnormal. The congenital abnormalities obtained in this way consisted of cleft palate, syndactylism, renal and pulmonary underdevelopment, and edema. Large supplements of pteroylglutamic acid to the deficiency diet permitted normal reproductive performance and prevented anomalies in the young. It seems that the teratogenic diet developed by Nelson, Asling, and Evans ('50, '52) when given from the tenth to the twenty-first day of gestation is the most effective teratogenic diet known at the present time.

Experiments with maternal dietary deficiency may be considered as a new tool of experimental embryology. In most studies of that science, eggs of fishes, amphibians, and birds have been used to produce morphologic changes by altering the chemical environment; advances in the science of nutrition have made possible the study of mammalian ova under varying chemical conditions, and it seems that only a beginning has been made in this new field. In the experiments described no attempt was made to simulate human dietary conditions, and it is unlikely that human congenital malformations can often be attributed to maternal dietary deficiency. Only in populations which are restricted in their food supply by isolation, customs, or violence, can conditions arise which are comparable to the experiments cited. Endemic cretinism caused by maternal iodine deficiency is a case in point, but it remains to be seen whether there are other comparable conditions which can induce congenital anomalies.

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[3] These investigators designate the day of finding sperm in the vaginal smear as day zero and the following day as day 1 of gestation.

Experiments of maternal dietary deficiency should have a permanent place in mammalian teratogenic research. In this connection it seems worth while to point out the relationship between malformations induced by maternal dietary deficiency and malformations of genetic origin. While these two kinds of malformations differ completely both in their origin and in their importance for the species, they do show points of similarity in their morphology and in their mechanism of development, which deserves to be emphasized.

By experiments with maternal dietary deficiency, types of congenital malformations can be induced which resemble, morphologically, malformations of genetic origin. Such imitations have been called phenocopies, a term coined by Goldschmidt ('38). Phenocopies are congenital nonhereditary malformations which duplicate malformations induced by mutant genes. For instance, cleft palate or syndactylism or coloboma of the retina may be of genetic origin; similar anomalies can be produced experimentally as nonhereditary phenocopies by riboflavin (Warkany *et al.*, '43b), pteroylglutamic acid (Nelson *et al.*, '50) or vitamin A deficiencies (Warkany and Schraffenberger, '46).

A second resemblance between effects of abnormal genes and effects of nutritional deficiencies is observed in the formation of syndromes of multiple anomalies. An abnormal gene frequently has a pleiotropic effect; it may cause malformations in a variety of organs which seem to be unrelated to each other in postnatal life. The Marfan syndrome and the Laurence-Moon-Biedl syndrome may be cited as examples from the field of human teratology and many similar syndromes have been described in rodents (Grüneberg, '47). Pleiotropic but nonhereditary effects have been observed as the result of experiments with maternal dietary deficiency. For instance, lack of vitamin A results in anomalies of the eye, of urogenital organs, and of the cardiovascular system. Nutrition experiments of this type result, therefore, not only in phenocopies of single organs but also in phenocopies of syndromes.

A third resemblance is that both a genic deficiency and a continuous nutritional deficiency act throughout the organogenetic period and that in both cases the embryo develops against a background which is permanently abnormal. In contrast are the acute teratogenic agents, such as the rubella virus or X rays, which attack the embryo but once and at a definite time. Genic and nutritional deficiencies are chronic abnormal states of prenatal life. They remain latent during some periods but become manifest in others when special demands are made by the embryo's metabolism (Landauer, '36).

Geneticists interested in teratology and in developmental genetics have long recognized the importance of the experimental production of phenocopies in the study of malformations of genetic origin. Dunn ('39–'40) has pointed out that the responses which a growing organism can make are directed into a limited number of channels and that environmental agencies may produce phenocopies by altering some developmental processes in the same way that gene changes or other mutations do. Landauer ('36, '40; Landauer and Bauman, '43) has used experimental induction of phenocopies to investigate the mechanisms by which the creeper mutation and rumplessness of fowl influence embryonic development. A knowledge of the abnormal channels opened up by modifying factors may throw some light on the mode of action of abnormal genes. Since little is known about the biochemical reactions of genes which lead to malformations, a knowledge of the reactions which lead to their phenocopies may be of value. Phenocopies induced by nutritional deficiencies can be attributed to a lack of well-defined chemical substances. Purines and pyrimidines which are synthesized with the aid of pteroylglutamic acid (Nelson *et al.*, '50, '52) as well as riboflavin are incorporated in prosthetic groups of enzymes. Some of these enzymes participate in biochemical reactions which determine the channels of embryonic development. Experiments of the type cited in this review indicate where biochemical reactions necessary for normal development can be blocked. It is possible that mutant genes are inadequate because they are unable to control the same critical reactions. It seems to me that in this field the geneticist and the nutritionist are approaching common ground and it is to be hoped that they will unite their efforts in the near future.

This paper began by presenting the results of experiments but factual reporting seems to have slowly drifted into hypothetical interpretation. The interpretations are open to criticism and dispute, but this may lead to a fruitful discussion.

DISCUSSION

L. B. RUSSELL: In connection with your comparison between the effects of X rays and of riboflavin deficiency, I am wondering whether the more widespread action of the X rays may not have been due to a high dose having been used? As we tried to show in our paper, increase in dose leads to an extension of responding period. Thus, in your experiment, irradiation with a high dose on the thirteenth day might be expected to yield not only the abnormalities most easily produced at that stage, but also others which, with a lower dose, would be elicited only by irradiation on the twelfth or fourteenth day. This idea finds support in the close resemblance of your radiation results for the thirteenth day to our summed results for days 10½ and 11½ (the mouse stages corresponding to your thirteenth and fourteenth days). It is therefore not impossible that, if you were to use a dose low enough to elicit only those changes most characteristic of thirteenth-day treatment, you might get a closer correspondence with the riboflavin-deficiency data. On the other hand, of course there may be (as we showed in our paper) real differences between the basic actions of the two teratogens.

WARKANY: I think it would be very interesting to attempt to produce the same syndrome by X rays and by riboflavin deficiency, as suggested by Dr. Russell. Whether it can be done, I do not know.

HICKS: Dr. Warkany and Dr. Wilson, in their published studies, emphasize something that has been very useful to all workers on the mechanisms of malformations from the medical point of view. That is, it is useful to think of these malformations as products of two factors — the *time,* which we have been emphasizing here, and the *agent.*

In my paper I tried to show, for example, that in the developing nervous or skeletal system the same lesions cannot be produced with acute hypoglycemia as with radiation or "radiomimetic" drugs.

Dr. Warkany and Dr. Wilson have shown other examples with dietary deficiencies although the duration of action of these procedures is a factor to be considered.

FURTH: One wonders whether there is a relation between vitamin deficiencies and irradiation. It is possible that irradiated animals, in general, are unable to synthesize vitamins or that their vitamin requirements are higher. More specifically, embryonal cells which have been slightly damaged are unable to use vitamins essential for their vital synthesis. How to demonstrate this is the problem. With animals having placenta, obviously this could not be done with ease. If irradiated cells are unable to utilize the vitamins (not simply take them up), the blood and tissue vitamin levels would probably be altered. I wonder whether, in animals without placenta, vitamin utilization studies might not be valuable. The opossum might be a suitable animal for such studies, but more than likely one would have to go to lower species.

There may be some blood-brain barrier with respect to protective substances available to cells of the central nervous system, and this may include substances needed for cell synthesis.

Chairman Weiss: I think I should like to reemphasize the reversal of an antero-posterior gradient in the susceptibility of the skeleton, where apparently after riboflavin deficiency, the posterior border is hit first so much more frequently than the anterior border that the ossification center in the marginal cases is dislocated toward the anterior border; whereas after X-ray treatment, I think you got exactly the reverse.

This type of reversal makes it almost impossible to explain the effectiveness of any of these agents in terms of particular susceptible phase; it is a very important observation.

Strehler: When vitamin A deficiency was supplemented, the figures indicate that approximately 53% of the animals studied possessed abnormalities on the unsupplemented diet — of which 17% involved cardiovascular abnormalities. On the other hand, in the 13-day supplemented material, some 25% of the animals showed abnormalities of which 70% were cardiovascular. If you multiply these numbers together you find that there is actually an increase in the number of cardiovascular abnormalities on a thirteenth-day supplemented diet.

Might not some caution be used in ascribing any of these particular abnormalities to the direct effect of any particular growth factor on the formation of the structures studied? Could it not just as well be something very indirect, some influence on some secondary product of the mother?

In relation to this, I wonder whether there is a general correspondence between the abnormalities that you observed in embryonic development and the abnormalities which such riboflavin deficiency, in particular, produces in young adults, e.g., lack of proper ossification, etc.

Warkany: We, too, have asked ourselves why there was an increase of cardiovascular anomalies when vitamin A was added on the thirteenth day. We think that some young with cardiovascular anomalies would have died in early intrauterine life without the supplement, and by giving the supplement, we saved some of them. This is one explanation, but we cannot prove it.

Whether riboflavin deficiency damages the embryo directly or whether the effect may be indirect and due to an injury to the mother remains an open question. I should not be surprised if a future need for reconsideration of the effects of riboflavin deficiency should arise. We consider these experiments as pilot experiments which require further study.

It should not be forgotten that our experiments were begun at a time when it was necessary to prove that congenital malformations of mammals could be produced by environmental factors. This was denied by many at that time, although it is generally accepted today. We have continued working along these lines but there is still much to be done. I think the reasons that more has not been done already is that nutritional teratogenic experiments are so tedious and disappointments are frequent.

Waelsch: I should like to comment on Dr. Warkany's distinction between chronic and the more immediate effects. I wonder whether one is justified in considering the effect of rubella, for example, and of X rays as definitely of the immediate and nonchronic type? To my knowledge, nothing is known about the mechanism of the rubella effect on developing human embryos, and I think the preceding discussions at this conference have shown that really very little is known about the mechanism of the X-ray effect. So I could well imagine that these effects are as ''chronic'' as some of the other effects, e.g., genetic or nutritional effects.

To come back to the discussion of the relative incidence of cardiovascular abnormalities in general, when dealing with a syndrome of abnormalities, it is very difficult to establish the relative frequency of the individual symptoms if you look only at newborn animals. The relative frequency varies throughout the embryonic period because there is prenatal death, i.e., selective prenatal death of embryos, with certain abnormalities, which would, of course, influence considerably the relative frequency of the individual symptoms of a syndrome.

GRUENWALD: I am glad to hear that the idea of the critical period being the only determining factor is losing ground. Particularly in the medical literature on rubella, there is considerable misuse of this concept when people claim that one thing happens during the second month, and another during the third month. The second month, after all, is not homogeneous. Human embryos at the beginning and at the end of the second month are quite different. I think it should be made clear that the specific influence of a given agent is in many instances more important than the exact time with which it acts, particularly because some of these agents apparently act for a long period of time.

Chairman WEISS: At the same time is it not a truism that if we choose to designate the period during which a tissue can be affected as the "critical" period, then that tissue can be affected only during the critical period? The question, therefore, is merely whether this "critical" period is the same for all agents, all stages, and all organs. Apparently, that is not the case, but there are still some critical periods.

GRUENWALD: We seem to come to the assumption, which is probably the only biologically correct one, that both factors act together; in some instances the critical period is more important and in some instances the agent is more important in determining just exactly what is going to happen. Would you agree to that?

Chairman WEISS: Not quite. I think the scientific procedure is simply to define what each partner in the process is doing. No biological system operates in a vacuum; it is always a cell reacting to something outside the cell. All we can do is simply describe this interaction. There is no point to the question as to which of the two is more important. If you take either one away, you get nothing.

SCHOTTÉ: I should like to attract attention to another phase of the problem of morphogenesis, the one which is encountered in regeneration. Students of regeneration, also, have elaborated a series of devices by means of which teratological interferences on morphogenesis can be studied. It is amazing to discover that, in regeneration, interferences with morphogenesis can be obtained which constitute a corollary to that which has been so exhaustively discussed here. These interferences are multiple. First, there are purely physical factors such as various manners of amputations which subsequently impose upon the regenerate changes in morphogenesis. Then there are interferences with hormones (thyroid, parathyroid, pituitary). Recently we were able to show that a critical period in respect to the action of the pituitary on the morphogenesis of the blastema exists: if the limb of a newt is allowed to regenerate for 5–7 days and then the pituitary is removed it has been shown that the subsequent morphogenesis of the regenerate is affected, but only for that period. In others words, if a limb is amputated prior to the removal of the pituitary, then club foot formations, syndactyly, brachymely, and other teratological interferences with the morphogenesis of the blastema result. However, the fundamental processes of regeneration, including growth and histogenesis, are not affected by the hormonal imbalance created.

With respect to the action of the nervous system it has been shown that the susceptible ''critical period'' of interference with morphogenetic processes is a different one. Singer has shown that the emancipation of blastemata from nerve influence takes place between the eleventh and fifteenth days. Dr. Brunst has also shown many years ago (1928) that the nervous system plays an important role in the creation of skeletal abnormalities. The same fundamental processes of morphogenesis, then, reveal themselves to respond differently in respect to the agents employed to test these processes.

So it is indicated, I think, in a conference devoted to the study of the action of radiations on growth and development, to suggest that the pioneer work which Drs. Butler, Brunst, and others have done with respect to the action of X rays on regeneration of limbs in amphibia be extended and correlated with the findings on mammalian embryogenesis which were presented at this conference.

Russell: I should like to reply to Dr. Waelsch's statement that one should not necessarily expect an acute effect from an agent which appears to have acute action, by pointing out that, on the other hand, one need not necessarily expect a chronic effect from an agent that appears chronic, such as a gene which is, after all, present from conception to birth. Actually, for many genes there are probably restricted periods for primary action which is called for by conditions arising in the course of differentiation.

In the general discussion on "critical periods," I should like to emphasize that this phrase is used by us merely to designate an end result, i.e., the developmental stages at which radiation has to be applied to produce a given change in character. The term certainly does not imply that the immediate primordium of the character malformed as a result of irradiation at a certain stage was damaged at that stage, i.e., during the critical period. The fact that different patterns of sensitivity may be demonstrated by the use of different teratogens is quite compatible with the concept of "critical periods" as used by us.

Warkany: I should like to state here why some of us are so concerned about the indiscriminate use of the term "critical period." I think all of us in this conference understand how complicated the establishment of "critical periods" is and that the term is interpreted differently by different people.

We are concerned about the overemphasis and misinterpretation of the idea of "critical periods" as used at the present time in the medical literature. There you find statements which indicate not only that there are critical periods for the origin of certain malformations, but it is also asserted that one can ascertain from a malformation the time at which the injurious agent acted.

This is not just a point of theory. Let us assume that the symptoms of a clinical syndrome can be attributed to arrest of development of several structures in the eighth week of fetal life. Does that permit the conclusion that the arrest of development is due to environmental interference at the time when the fetus was eight weeks old? I doubt that anybody here will draw such a conclusion, but there are statements to that effect in the recent medical literature. Mongolism, esophageal atresia, and ''retrolental fibroplasia'' have been attributed to maternal disorders at a certain date of pregnancy, because the morphology of the anomalies can somehow be correlated with embryologic events at that date.

We have to make it clear that such conclusions cannot be drawn. It has been stated, for instance, that a persistent ostium primum in mongolism indicates that the septum primum and the endocardial cushions developed adequately until about the seventh week, when they were arrested just short of fusion. This is not correct, since the decisive arresting factor may have acted much earlier; yet the interference does not become recognizable before the eighth week. The best example in this respect is the open ductus which may result from maternal rubella in the first two months of pregnancy. The ductus fails to close at birth or after birth, but the injurious agent acted seven months earlier. The ''critical period'' is not the time when the ductus fails to close, but the time of a much earlier stage of development when an injury to the vascular system predetermines the persistence of the ductus. Although the anomaly does not ''appear'' until birth, the damage is done much earlier.

The morphology of congenital anomalies does not permit us to draw definite conclusions as to their date of origin. There are practical considerations involved. If the simplified idea of ''critical periods'' should reach the lay public we must expect serious consequences. If, for instance, a woman who had an automobile accident in a taxicab during the seventh week of pregnancy, gives birth to a mongol child, she could sue the taxi company. This will happen when we let this erroneous idea of critical periods become too widespread.

Hicks: On the other hand, a malrotation of the gut of cyclopia or an encephalocele could not happen as a result of a birth injury.

Warkany: I think the embryologic time table can be used to state that a malformation must have been caused *before* a certain time but one cannot say how early before the ''critical period'' the injurious agent acted; but even this may not always be possible since congenital malformations may be caused by secondary destructive processes after an organ had been normally formed.

Waelsch: I should like to say just one word in response to Dr. Russell's remark. I do not think it is justified to call the effects of a gene, as you did, acute just because it has not been demonstrated to be ''chronic,'' in quotation marks. You said one would expect to be able to demonstrate its effects in tissues other than the ones that are obviously affected. The failure to demonstrate it is not necessarily a proof that the effect is acute and not chronic. It may be just a problem of method and knowing what to look for in the effect of a gene.

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THE EXPERIMENTAL PRODUCTION OF CLEFT PALATE WITH CORTISONE AND OTHER HORMONES

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ONE FIGURE

Experimental mammalian teratology is rapidly establishing itself both as a method of embryological analysis and as a means of gaining understanding of the causes of congenital defects in humans. Hormonal aberrations (along with viruses, nutritional deficiencies, and hypoxia) might logically be suspected of being causally associated with "spontaneously" occurring congenital defects, since they are one of the hazards that a woman may actually face during pregnancy. It is therefore somewhat surprising that, until recently, hormones have received relatively little attention as teratogenic agents. The discovery in our laboratory that cortisone is teratogenic in mice (Fraser and Fainstat, '51) raises the question of whether this is so in man.

REVIEW OF LITERATURE

Before discussing our work on the teratogenic effects of cortisone the present paper will review briefly the studies of others on the embryonic effects of various hormones given to pregnant mammals.

Pituitary hormones. Due to the limited availability of pituitary hormones for research, little has been done on their teratogenic properties. Steiniger ('40) provided an interesting example of how an environmental agent can potentiate the effects of a genetic susceptibility. He showed that injection of anterior pituitary extract into pregnant female mice of a strain showing a 10% incidence of spontaneous cleft lip and palate raised the incidence to 17% in the offspring of treated mothers. However, anterior pituitary extracts containing growth hormone had no teratogenic effects on the offspring of pregnant rats when injected about the fourteenth gestational day (Barnes and Swyer, '52).

Jost ('50, '51, '52), in an intriguing series of experiments, has shown that pitressin, injected directly into rat or rabbit embryos causes the appearance in their extremities of edema, and subcutaneous blebs of clear fluid, followed by extravasation of blood, necrosis, and sloughing of tissue, and leading to a variety of defects in the embryonic limbs and tail. The effect varies with the dose of drug and the age of the fetus. Pitressin given in much larger doses to pregnant females also produces these embryonic changes *(ibid.)*. Adrenalin (Jost, personal communication) and, to a less extent, cortisone given directly to the embryo produced similar changes. This work supports the suggestion that local ischemia may be the cause of certain types of congenital defects (Lombard, '50). Jost does not mention seeing cleft palates in any of his treated embryos.

Thyroid. Little is known, experimentally, of the embryonic effects of excessive amounts of maternal thyroxine. Giroud and de Rothschild ('51) were able to produce cataracts in the offspring of pregnant rats fed thyroxine, but no other defects were seen in these animals.

Pancreas. The elegant work of Landauer and Rhodes ('52) and of Duraiswami ('52) on the teratogenic effects of insulin on chick embryos will be reviewed by another participant in this Conference (Landauer) and will therefore not be discussed here. Insulin can also be teratogenic in mammals, causing skeletal and possibly other defects in the offspring of rats treated with insulin throughout most of the pregnancy (Lichtenstein *et al.*, '51). Whether the defects caused in mice by alloxan (Ross and Spector, '52) are the result of an insulin deficiency, or due to some other metabolic upset in the mother, or to a direct toxic effect of alloxan on the embryo is not yet clear.

Sex hormones. Many workers have studied the effects of sex hormones injected into the mother on the development of the genito-urinary system of the embryo, but little attention has been directed to effects on other parts of the embryo (Moore, '50).

Adrenal. Several workers have reported that cortisone, given to pregnant rats, rabbits, or mice, has deleterious effects on the embryos, including intrauterine death, abortion, resorption, reduction in embryonic size, and reduction in size of the embryonic adrenals (Courrier *et al.,* '51; Courrier and Colonge, '51; Robson and Sharaf, '51; Glaubach *et al.,* '51; DeCosta and Abelman, '52). Similar effects were observed with ACTH (Robson and Sharaf, '51). Cortisone injected directly into the embryo had no effect on fetal weight but frequently killed the embryo (Leroy and Domm, '51). Neuroblast damage was reported by Hicks ('52) in the offspring of pregnant mice given cortisone, ACTH, or a variety of other agents. None of these workers reported cleft palates or other gross congenital defects in the offspring of treated animals. It is hoped that other workers will attempt to confirm the findings presented here and in previous publications (Fraser and Fainstat, '51; Kalter and Fraser, '52; Fraser, '53; Fraser *et al.,* '53) concerning the teratogenic production of cleft palates and other congenital defects by the injection into pregnant mice of cortisone and related compounds.

MATERIALS AND METHODS

The results to be presented were obtained with two strains of mice and the descendants of crosses between them. These are:

(1) A/Jax, inbred brother-sister for 88 generations; an albino, high mammary carcinoma strain, with a 5% incidence of spontaneous clefts of the alveolar margin and palate. A few animals from a subline of strain A inbred in this labora-

tory for eleven generations have been included in this group. Their reaction to the teratogenic effects of cortisone appear identical with those of strain A/Jax.

(2) C57BL/6 Jax, inbred brother-sister for 45 generations; a black, low mammary carcinoma strain with a 0.2% incidence of spontaneous clefts of the alveolus and palate.

In previous reports the gestational stage was measured either by counting forward from the time of observation of a vaginal plug (a sign that copulation has recently taken place — Grüneberg, '52) or by counting backward from the time of birth, assuming a gestational period of 19 days. Accumulating data on pregnancies timed from the observation of a vaginal plug suggest that the mean length of gestation in the two inbred strains used is 20 days, but that in hybrid females it is 19 days. Therefore, unless otherwise stated, the timing of treatments in the data to be presented is based on this assumption. In the embryological studies, where timing was particularly important, females were mated only in proestrus or estrus in the evening, and separated from the males the following morning.

The cortisone used in these experiments was the Merck "Cortone^{R}" saline suspension of cortisone acetate, 25 mg/cc, with added suspending agents and 1.5% benzyl alcohol. The compound F acetate (17-hydroxy-corticosterone-acetate) was also a Merck preparation, of the same concentration, in a similar vehicle. The ACTH was produced by the Armour Laboratories, and was dissolved in saline before being injected. Injections were given intramuscularly in the flank.

Early in our experiments it became evident that there were marked strain differences in reaction to cortisone as manifested by the incidence of cleft palate in the offspring of treated females. It was found empirically that a dose of 2.5 mg of cortisone given daily for 4 days was suitable for demonstrating such differences (giving high values for strain A and low values for C57BL without causing too many resorptions), and this standard dose has been used in all the experiments discussed here.

EXPERIMENTAL RESULTS

Control data. Cleft palate of the type induced by cortisone (i.e., without cleft lip) has not occurred in 164 strain A or 140 strain C57BL offspring of untreated mothers. Ingalls *et al.* ('52) did not observe isolated cleft palates in any of 1180 control embryos observed during his experiments with anoxia, and to the writer's knowledge this defect has never been reported to occur spontaneously. It has further been shown (Fraser and Fainstat, '51) that injections into pregnant mice of the inert vehicle used to suspend the cortisone had no visible effects on the developing embryo. It can therefore be safely concluded that the cleft palates observed in the offspring of treated females were due to the treatment.

TABLE 1

Resorption of litters in pregnant females given 2.5 mg of cortisone per day for 4 days (all crosses)

DAY TREATMENT STARTED	NO. OF FEMALES TREATED	NO. RESORBED	PERCENTAGE RESORBED
7	17	15	88
8	37	19	51
9	63	20	32
10	106	27	25
11	101	13	13
12	103	1	1
13	63	7	11
14	46	4	9
15	26	0	0
16	16	1	6
17	22	0	0
18	16	1	6

Embryonic effects of cortisone. Table 1 shows that cortisone given early in pregnancy caused resorption of litters in a high proportion of cases (88% of those in which treatment started on day 7), but that the later the treatment was started the fewer resorptions occurred (data from all crosses have been lumped in this table since no obvious strain differences could be seen in the frequency of cortisone-induced resorption of litters). It has also been shown that the proportion of stillborn offspring increased with increasing dosage, and that doses of cortisone too low to cause cleft palates (0.625 mg × 4 days) still caused an appreciable number of stillbirths. Teratogenic doses of cortisone also caused a reduction in litter size, and a reduction in weight of the newborn offspring of treated mothers (Fainstat, '51).

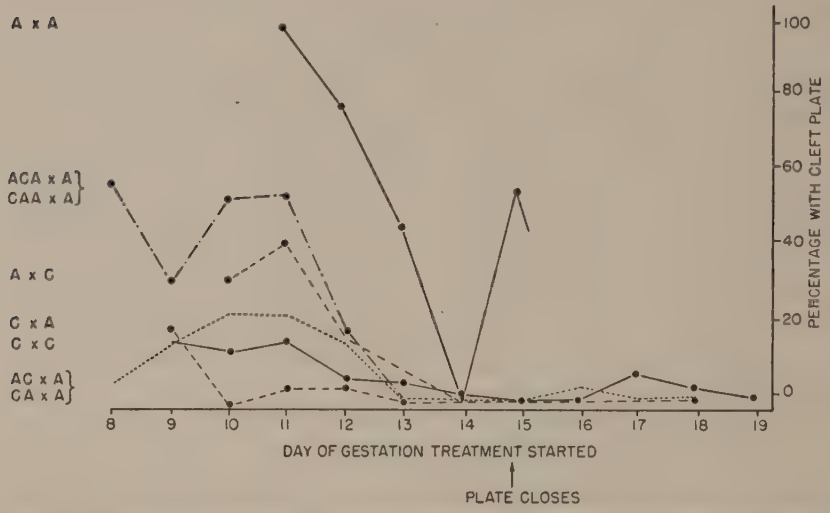

Fig. 1 Frequencies of cleft palate in offspring of mothers of various genotypes given 2.5 mg of cortisone daily for 4 days, beginning at various gestational stages.

Figure 1 illustrates the frequency of cleft palate in various genetic situations and its relation to the gestational stage at which cortisone injections were begun (the data on which the graph is based will be published in detail elsewhere). It can be seen that the incidence of cleft palate is highest when treatment is started on day 10 or 11, and decreases when treatment begins later than this. There are also small secondary peaks occurring when treatment begins after the palate normally closes (day 15).

Genetic analysis. Table 2 presents the frequencies of cortisone-induced cleft palate in various crosses when treatment was started on day 11 (the stage when strain differences were most pronounced and for which the data are most abundant).

What is the genetic basis for the pronounced strain difference demonstrated in the first two rows of table 2, where strain A shows a 100%, and strain C57BL a 17% frequency of cleft palate in the offspring of treated mothers? Rows 3 and 4 show the results of crossing the two strains, treating the pure-strain mothers and measuring the frequency of cleft palates in the F_1 hybrid offspring. A distinct reciprocal cross difference is apparent, since 43% of the hybrid offspring of strain A mothers have cleft palates as compared with 4% of the genetically similar hybrid offspring of C57BL mothers. Such reciprocal cross differences may be interpreted in one of three ways. Either the factors responsible for the strain difference are transmitted through the cytoplasm and act in the embryo, or they are transmitted through the cytoplasm and act in the mother, or they are transmitted through the germ plasm and act in the mother.

TABLE 2

Cortisone-induced cleft palate in offspring of pregnant females given four daily doses of 2.5 mg of cortisone beginning on day 11

CROSS	NO. OF FEMALES TREATED	NO. OF OFFSPRING	NO. WITH CLEFT PALATE	PERCENTAGE WITH CLEFT PALATE
A × A	10	36	36	100
C × C	22	86	15	17
A × C	11	46	20	43
C × A	12	82	3	4
AC × A	15	116	26	22
CA × A	11	71	18	25
ACA × A	5	40	24	60
CAA × A	9	51	27	53
	Total	528	169	

The results of backcrossing the two types of hybrid females to strain A males (treating the hybrid mothers and measuring the incidence of cleft palate in the backcross offspring) will distinguish between cytoplasmic and nuclear inheritance of the factors responsible for the strain difference. The two types of hybrid females will be identical in their nuclear heredity. So will the two types of backcross offspring. But the two types of hybrid females will differ in their cytoplasmic factors, since the offspring of cross A × C57BL (referred to as AC) will receive type A cytoplasmic factors, and offspring of the reciprocal cross, CA, will receive C57BL type cytoplasmic factors. These differences will be transmitted to the backcross offspring.

Therefore, if cytoplasmic factors are involved, the frequencies of cleft palate in the offspring of hybrid females backcrossed to strain A males will be higher in the cross AC × A than in the cross CA × A. On the other hand, if nuclear factors are involved, results for the two types of backcross should be identical. Table 2, rows 5 and 6, shows that the results are indeed virtually identical (22% for AC × A and 25% for CA × A). It must be concluded, therefore, that the factors responsible for the reciprocal cross difference in reaction to the teratogenic action of cortisone reside in the genotypes of the pure-strain mothers.

On the other hand, one cannot ignore the effect of the embryo's genotype. The F_1 offspring from strain A mothers have a much lower frequency of cleft palate (43%) than the pure strain A offspring (100%) although they both have the same sort of mothers. Furthermore, the frequency of cleft palate in the F_1 hybrids of C57BL mothers (4%) is lower than that in the pure-strain C57BL mothers (17%). These differences are statistically significant and may be attributed to "heterosis," although naming the phenomenon does not contribute to our understanding of it.

To gain more information about the genetics of the strain difference in reaction to cortisone, the female offspring of the first backcross were mated to strain A males (ACA × A and CAA × A). Again there was no significant difference in the frequencies of cleft palate for the two types of backcross (table 2). The combined value for both crosses, with treatment beginning on day 11, was 56%. Since the character investigated (frequency of cortisone-induced cleft palate) is influenced both by the maternal and fetal genotype it is very difficult to interpret this result. Our only conclusion is that, since repeated backcrossing to the strain A males causes a relatively slow rise in the incidence of cleft palates in the offspring of treated mothers, the genetic situation is probably polyfactorial.

This impression is borne out by a consideration of the variability among different females from the same cross. Although it is difficult to obtain a sufficient number of offspring from any one female to estimate reliably her susceptibility to the teratogenic effect of cortisone, the results indicate that there is much more variability among the backcross females than among the pure-strain or F_1 hybrid females. This is what one would expect in the transmission of a quantitative character (Kalter, '53).

Teratogenic effects of compound F and of ACTH. The discovery of the teratogenic effects of cortisone raised the question of whether congenital defects in humans could be caused by excessive secretions of the adrenal cortex. This led to an investigation of the teratogenic effects of compound F, which showed that this hormone had effects on the embryonic mouse palate similar to those of cortisone (Kalter and Fraser, '52; Kalter, '53).

Even more important was the question of whether the animals own adrenals were capable of producing enough cortisone-like hormone to have teratogenic effects. If so, one would expect that cleft palates could be produced by injecting ACTH into pregnant mice. Our first trials with this hormone gave inconsistent results, perhaps due to the variability in potency between different lots of the drug, or to the difficulty of maintaining effective levels in the blood stream for prolonged periods of time. The litters were either born with no obvious congenital defects, or were aborted. However we have recently observed three litters from ACTH-treated strain A females containing a total of sixteen offspring of which nine had cleft palates. The mothers had received 5 mg of ACTH, every 6 hours, for 2 or 3 days, beginning on the thirteenth gestational day (Fraser, Heiberg, and Kalter, unpublished). Since cleft palates have never been observed in the offspring of untreated mothers, we feel that there is no reason to doubt that, unless ACTH acts directly on the embryo, it can cause the adrenal cortex to secrete teratogenic amounts of cortisone.

Embryological studies. One of the major difficulties in analyzing the embryogeny of a congenital defect is the fact that one cannot usually be sure that any given embryo, removed for study prior to the appearance of the defect, would actually have had the defect if allowed to continue development. Our ability, under the appropriate conditions, to produce cleft palates in 100% of the offspring of treated females provides a unique opportunity to study the pathogenesis of this defect; we can be reasonably sure that an embryo removed from a suitably treated mother on, say, the fifteenth gestational day, would have had a cleft palate at birth.

For our embryological studies, strain A females shown by vaginal smear to be in estrus or proestrus, were placed with strain A males overnight and separated from them the following morning. Since ovulation usually takes place at or shortly after midnight (Grüneberg, '52), estimates of gestational stage have been based on the assumption that conception occurs at 2 o'clock A. M.

Although our studies on this phase of the project are in only their preliminary phases, certain tentative conclusions may be drawn. In the normal embryo, early on the fourteenth gestational day, the palatine bars are hanging in the sagittal plane, i.e., at right angles to the plane they will occupy in the completed palate. Late on the fourteenth day, the palatine bars rotate medially, somehow getting around the intervening tongue, until their distal margins meet in the midline, and fuse. This is a fairly rapid process and is completed early on the fifteenth gestational day. [There is evidence (Lazzaro, '40) that a similar process occurs in other species including human beings.] Subsequently there is a process of lateral growth in keeping with the enlargement of the head in general.

In embryos of cortisone-treated mothers there seems to be no deviation from the normal path of development until the fourteenth day, although treatment is begun on the eleventh day. The first observable effect of the cortisone treatment on the embryonic palate seems to be a delay in the process of rotation of the palatine bars. The degree of delay is rather variable. The palatine bars in treated embryos may reach the plane of the completed palate soon enough so that their medial margins very nearly touch. However the process of lateral growth, occurring as the head enlarges, draws the palatine bars away from each other and the width of the cleft enlarges as the embryo grows older (Walker, unpublished).

TABLE 3

Cleft palate induced by cortisone given after palate normally closes (including all strains and all dosages used)

DAY	NO. OF FEMALES TREATED	NO. OF OFFSPRING	NO. WITH CLEFT PALATE	PERCENTAGE WITH CLEFT PALATE
16	19	98	3	3
17	32	185	6	3
18	19	110	2	2
19	3	18	0	0
Total	73	411	11	3

No embryological studies have as yet been done on embryos from mothers treated after the palate normally closes, although cleft palates have occasionally been observed in such offspring (table 3). It seems highly unlikely that these could be due to errors in timing, since the offspring in each case had the external morphological characteristics of full-term animals. It must therefore be concluded that either the cortisone treatment can cause a regressive change in the palate, leading sometimes to a cleft, or, occasionally, the time of palate closure is greatly delayed so that the palate is still open when treatment begins, late in the gestational period. The latter possibility seems unlikely but has not yet been rigorously excluded.

CONCLUSIONS

It has been clearly established that cortisone is teratogenic in mice. Its effectiveness in causing cleft palate varies with the dose of drug and the gestational stage treated. This relation has been noted by other workers for other teratogens (Ingalls *et al.*, '52; Russell, '49, '50; Haskin, '48; Warkany and Schraffenberger, '47). We have further shown that there are marked genetic differences in reaction to the teratogenic effects of cortisone, and that the final result depends partly on the genotype of the mother and partly on that of the fetus.

There is some evidence that congenital palatine clefts may be caused by two different mechanisms, one acting well before closure of the palate and causing delay in rotation of the palatine bars, and one causing a regressive change in the palate after it has closed.

There is also evidence that the adrenal cortex of the pregnant female may be able to produce enough cortisone to interfere with the development of her embryos. This is suggested by the fact that compound F [thought to be the naturally occurring adrenal glucocorticoid (Conn *et al.*, '51)] is teratogenic, and more strongly by the fact that maternal treatment with ACTH causes cleft palates in the embryos.

It is interesting that the highest frequency of cortisone-induced cleft palates is obtained when injections are begun on the eleventh gestational day, 4 days before the palate normally closes, whereas ACTH seems to be most effective on the thirteenth day, judging from the small amount of data at hand. (No data are yet available for day 14.) This might be due to the fact that cortisone is absorbed more slowly than ACTH, and takes longer to attain an effective level in the bloodstream. If so, our findings would be fairly consistent with those of Ingalls *et al.* ('52) who found that anoxia caused cleft palates most frequently when applied on day 14.5. On the other hand, Russell ('49) and Russell and Russell ('52) working with X rays, found the highest frequency of cleft palates in the offspring of mice irradiated on the tenth or eleventh gestational day, and Warkany ('44) found that maternal riboflavin deficiency caused cleft palates in rat fetuses through some process occurring on the thirteenth gestational day, 5 days before the palate normally closes. A similar critical period was noted in rats treated with nitrogen mustard (Haskin, '48). Nelson *et al.* ('52) found that a maternal pteroylglutamic acid deficiency caused cleft palates in a high proportion of fetal rats if treatment was instituted on the tenth or eleventh, but not on the thirteenth day of gestation. It appears that, for several teratogens, failure of the palate to close at the normal time is determined by events that happen well before this time.

The demonstration that maternal treatment with ACTH can cause congenital defects in the offspring suggests that stresses of various sorts might be teratogenic. This leads one to wonder whether other known teratogens might be acting simply as stressors, causing the adrenal to secrete excessive amounts of cortisone. Experiments with teratogens and adrenalectomized pregnant females might help to decide this question.

What is the significance of our findings in relation to human congenital defects? For one thing, they might draw attention to the importance of the genetic constitution in deciding which individual will be affected, even when a definite environmental agent such as rubella has been shown to cause congenital defects. Congenital defects, apart from those showing regular Mendelian inheritance, cannot be attributed either wholly to genetic or wholly to environmental causes, but are the result of an interaction between the two. The problem is further complicated by the fact that genetically identical embryos, in the same uterus, do not necessarily react similarly to the same extrinsic agent. There must be an intrauterine variability that causes either differences in the amount of teratogen reaching the fetus or differences in the fetal reaction to it. [Similar conclusions can be drawn from studies of diseases in twins (Metrakos, '53).]

An additional feature of our findings is the discovery that the genetic constitution of the mother as well as that of the embryo plays an important role in determining whether a teratogenic agent actually succeeds in causing a congenital defect. This may reorient our thinking when trying to identify relevant genetic factors in the etiologies of congenital defects in human beings (Fraser, '53).

Finally, there is the question of whether cortisone, administered therapeutically or produced in excess following a stress, might be teratogenic in humans. The dose per unit of body weight used in our experiments is tremendous in comparison to those used therapeutically. Yet there are marked interspecific differences in reaction to cortisone, and the pregnant mouse seems to be highly resistant to the dose used, showing no grossly obvious deleterious effects other than a slight, transient weight loss.

The suggestions that emotional stress during pregnancy may lead to congenital defects (Klotz, '52; Levy-Solal *et al.*, '50) must be regarded very cautiously, since it is extremely difficult to collect convincing data on this point without doing a prospective study.

Thiersch ('52) has demonstrated that it is possible for an agent known to be teratogenic in rats (4-aminopteroylglutamic acid) to cause developmental aberrations when given to pregnant human females. Whether the same is true of cortisone can be decided only by observing the results of treating pregnant human females with cortisone. No defects attributable to cortisone have as yet been observed (DeCosta, '52; Christensen *et al.*, '52) but very few women have been treated with cortisone during the second month of pregnancy, the embryonic stage in which many congenital defects are thought to be initiated. Even if cortisone is teratogenic in humans, it may be expected to produce defects only when given during the appropriate gestational stage to a mother and fetus with susceptible genetic constitutions. In view of this, a large series of cases will have to be observed before it can safely be concluded that cortisone, given to the pregnant mother, is not harmful to the human fetus.

SUMMARY

The production of cleft palates in the offspring of pregnant mice treated with cortisone has been analyzed genetically. The genetic basis for susceptibility to the teratogenic action of cortisone is polyfactorial, depending partly on the genotype of the mother, and partly on that of the embryo.

Injection of pregnant mice with ACTH also leads to the production of cleft palates in the offspring, although less predictably than with cortisone. This suggests that the adrenal cortex of the pregnant female may be able to produce teratogenic amounts of cortisone.

Embryological analysis shows that cortisone interferes with closure of the embryonic palate by delaying the rotation of the palatine bars from the sagittal to the transverse plane.

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DISCUSSION

Chairman Dunn: Dr. Fraser reminds us at just the right time that there are always two parties to changes of this sort. In experimental teratology, the responding and irritating systems are of equal importance. Now we have three systems to take into account: the responding systems of the mothers and the embryos, and the agent.

Runner: I should like to submit some general confirmation of the work of Dr. Fraser on experimental production of modified differentiations in the mouse. Dr. Fraser used cleft palate as an end point, whereas we selected the frequencies with which spontaneous and induced anomalies occur within inbred strains of mice. Among other things, we treated pregnant mice with smaller amounts of cortisone than did Dr. Fraser.

We first applied a battery of six experimental treatments to animals having a uniform inheritance. A single genotype (inbred strain) subjected to different treatments showed that differentiation of vertebra number 26 was altered by five out of six treatments. This demonstrated a generalized susceptibility of this character to prenatal insults. Second, we applied a single influence on animals from a battery of five hereditary backgrounds. The experiment induced alteration of twenty-six morphological characters, with more or less regular frequency. Of these, 80% occurred spontaneously in low frequency in the corresponding inbred strains. The pattern with which anomalies were induced was characteristic for each hereditary type.

Our observations agree with those of Fraser that the types of anomalies which are experimentally induced are at times more dependent on inherited susceptibilities of the embryo than on the experimental treatment employed.

CARTER: In your experiments, strain A gave a high cleft-palate response to cortisone. In many sublines of this strain cleft palate and harelip occur spontaneously in up to about 10% of the young; the development of this condition has been investigated by Sheldon C. Reed and others. Do you consider that strain A is abnormally sensitive to developmental upsets of the palate?

FRASER: In our laboratory, strain A produces a 5% incidence of harelip with cleft palate; and we have excluded all harelip animals from our data. The incidence of harelip does not seem to be increased by the cortisone treatment.

Whether there is a common basis for the underlying genetic susceptibility to clefts of both the lip and palate we cannot say. It is certainly true that we get a high percentage of cleft palates in DBA, which to my knowledge rarely produces clefts spontaneously, and an appreciable percentage in C57BL, in which I think I have seen only two harelips altogether, a frequency of 0.2%.

HICKS: Our experiments with cortisone, ACTH, and several steroid hormones administered to pregnant and newborn animals may expand the picture of what cortisone may do to the developing animal. The palate is not the only vulnerable part of the fetus or newborn. Large doses of cortisone or ACTH damage primitive neuroblasts and spongioblasts, alter the rate of growth, and strongly inhibit hair follicle growth. The early embryo has been unaffected, however.

It is worth emphasis that most of these things we do in the laboratory with hormones on growing animals are the result of large doses, usually larger than those attained in clinical practice.

Chairman DUNN: Have you tried to overcome the threshold of the more resistant strain by increasing the dose?

FRASER: No, sir, not yet.

RUSSELL: Is the cleft palate induced by treatment on day 17 of the same type as that induced by earlier treatment?

FRASER: We have not studied the embryological stages of the late treated ones as yet. Superficially, they look the same.

RUSSELL: I was just wondering how the formation of the abnormality could be completed in the short interval between treatment and birth?

FRASER: I could not tell you.

WAELSCH: Did I understand you to say that you think the mechanism responsible for harelip and that responsible for cleft palate are separated either in time or in some other aspect?

FRASER: I think that is a legitimate assumption.

WILSON: I am interested in Dr. Fraser's suggestion that internal stress might be involved in producing this type of result. We have been interested in the same subject and have attempted to put the idea to a test, particularly the question of maternal physiologic stress. Perhaps that is not the type of stress you had in mind, but we have tried to look into the matter of how severe a physiological alteration or insult to the mother could be tolerated by the embryo. One such maternal stress was hemorrhagic anemia at various times during pregnancy. So far, anemia of such severity as to kill at least half of the pregnant mothers caused a negligible effect in the offspring. Likewise, poisoning with carbon tetrachloride sufficient to kill 60% of the mothers did not affect the surviving young.

GRUENWALD: We should distinguish between stress of the mother and stress of the fetus. Of course we do not know to what extent the fetus can have an orderly reaction to stress. As far as the human is concerned, it has been suggested that, in prematurely born infants, the pituitary is not yet able to secrete enough ACTH in response to stress, whereas in full-term infants it does. I think some of this work was repeated in rats. To what extent that holds for mice, I do not know. If there is such a thing as malformations produced by stress, we should eventually arrive at a variety of noxious agents, such as Dr. Wilson has tried, which will produce the same effects; the effect of stress on the adult, shown by Selye and others, is rather nonspecific.

FRASER: I do not think we can say yet, but we may be arriving at such a point. There is no evidence that a variety of agents may not produce similar defects if the genetic component is controlled. Failure to obtain a given defect with a given form of stress in a given animal does not mean that it might not be obtained in a genetically susceptible animal exposed to the same stress.

HICKS: This palate lesion developing after the palate forms may be an example of the sort of thing Dr. Gruenwald spoke of; namely, a malformation resulting from destruction of a tissue already formed, rather than one in the process of growth.

The histology of this change should be very interesting. When will it appear?

FRASER: In the near future, I hope.

WARKANY: There is one report in the literature on human cleft palate (Politzer, '37) in which the author, an embryologist, concluded that a cleft palate can originate soon after fusion of the palatine processes by secondary destruction. I think that, in one of your slides in which the palatine processes of a treated animal were shown in cross section, they looked smaller than the normals. Could cortisone destroy mesenchyme or the connective tissue which makes up the primary palate and, in this way, cause cleft palate? If this is true, cleft palate could arise by cortisone injection even after the primary closure of the palate.

FRASER: We were not able to decide whether the smaller appearance of this bar was due to the cortisone acting on it or merely that it had been hanging there for a day after it normally should have been up and pulled centrally by the other bar.

GRUENWALD: Dr. Fraser mentioned that the growth of the head apparently has a tendency to pull these palatine processes apart. If that is a fact, it might in some instances keep the palatine processes from joining in the first place; and in other instances pull them apart again after they had joined. This might explain primary and secondary clefts by similar host mechanisms.

Chairman DUNN: One aspect of the late-occurring change: On what days were the treatments given? This is simply to remind us of the amount of lag between the treatment and the appearance of the later form of cleft palate.

FRASER: We had some when treatment started on the sixteenth, seventeenth, and eighteenth days.

Chairman DUNN: From the sixteenth-day treatment, when was the abnormality observed? I am trying to locate the lag.

FRASER: All these were observed at birth. We do not know how long before this they occurred.

SCHOTTÉ: I have recently obtained evidence that mesenchyme cells in the form of blastema cells can be destroyed by cortisone. I have seen the blastema regress and disappear if the injections are performed when amputated limbs are at the stage of young blastema.

Chairman DUNN: I think it was brought out in the earlier discussion that perhaps there are two forms, one of which involves changes in the alveolar margin, and the other not. Do you consider these two to be separated in time of origin?

FRASER: We know from the embryological work of Reed that the one with the cleft lip happens earlier.

WEISS: Dr. Schotté, you say that it destroys the connective tissue cells. Do you see pycnotic cells; or does it destroy the connective tissue fibers?

SCHOTTÉ: It has only transformed an existing blastema about 12 days old into a thick layer of connective tissue; so I am not yet really sure.

WEISS: I do not mean to object, but merely point out that we must be very careful about identifying the point of action of cortisone, whether it affects the cells or the intercellular matrix.

SCHOTTÉ: I am not actually sure of the mechanism of its action. I can only say that after the injections, the cells of the regenerate have ceased to be blastematous in appearance and that they have become ordinary dermis or connective tissue.

WEISS: The mechanism of snapping shut of the palatine bars which was mentioned sounds like a connective-tissue mechanism. I am wondering whether that is really the point of attack of the cortisone in those cases. Have any studies been made of the fibers, either cytochemically or electronmicroscopically?

SCHOTTÉ: I do not know.

MILLER: In regard to the "snapping up" of the lateral palatine processes, it appeared from the lantern slides that there might possibly be another interpretation of the data. In the stage in which the jaw has dropped, allowing the tongue to slip out from between the palatine processes, the tongue is flattened dorsoventrally as if it might be exerting an upward pressure on the two lateral palatine processes. You have ruled out this possibility?

FRASER: Perhaps I should emphasize again that we have been working on the embryology for only a few months; and as I said in my talk, this is based on a very small body of data. We did see one animal where my colleague, Mr. Walker, could pull the tongue down and actually see the bar snap up, and then he was able to push the bars down again and get the tongue back in between them, and it looked just like it had in the first place. Then the tongue could be pulled down again and the bars would snap up. But that is only one animal. I would not want to state this as an established fact until we have given it further study.

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ON THE CHEMICAL PRODUCTION OF DEVELOPMENTAL ABNORMALITIES AND OF PHENOCOPIES IN CHICKEN EMBRYOS [1]

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SEVEN FIGURES

Interest in experiments aiming at the production of developmental abnormalities by chemical means and at their epigenetic analysis has greatly increased of late. The comprehensive work of Paul Ancel has contributed much to this rejuvenescence of experimental teratology, especially as it is concerned with the chicken embryo. Observations on embryos relating to the effects of nutritional deficiencies and metabolic antagonists have brought to light many important facts. Biochemical determinations on tissues and organs of embryos have given a first glimpse into the metabolic complexities of differentiation, but have also provided reassurance that such analyses may be rewarding.

This review will be confined chiefly to one phase of our work at Storrs, viz., experiments with 4- and 5-day chicken embryos (Landauer, '45 to '53; Zwilling, '48 to '51). Interesting problems are raised by work on earlier stages of development, but these will only be touched upon insofar as such observations help to elucidate the results obtained in later stages. One reason for this limitation is that our findings confirm the conclusion of Ancel ('50) that, as development of the embryo proceeds, its parts acquire more specific vulnerability to treatment with particular teratogenic compounds. Hence the chances improve for a successful analysis and interpretation.

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The fact that, with chemical methods, it is possible to produce syndromes of malformations which bear a close resemblance to certain mutant conditions and which for this reason have been referred to as phenocopies appears to the student who hopes to penetrate the mysteries of developmental genetics. In this respect, the later stages of experimentation also offer greater hope for arriving at useful conclusions.

The observations to be reviewed, unless specified otherwise, were incident to experiments in which certain compounds, usually dissolved in saline, had been injected into the yolk sac of developing chicken eggs.

MORPHOLOGICAL CONSEQUENCES OF TREATMENT: SIMILARITIES AND DISSIMILARITIES

The malformations produced by each teratogenic substance vary quantitatively (in incidence) and qualitatively (in morphological expression) with dosage and various other factors to be discussed subsequently. For the present it will suffice to give a brief survey of the more important features characterizing the syndromes that are brought on by injection at 4 or 5 days of incubation of the compounds under discussion (table 1).

The principal seat of abnormalities is the skeleton of the face and of the extremities. The ''parrot-beak'' condition, the features of which are a slight shortening of the lower beak associated with a downward curvature of the upper beak, is found after treatment with sulfanilamide, eserine, and thallium. It is also fairly often encountered after the injection of insulin, but, in our experience, a shortening of the upper beak is the more common facial defect produced by insulin. After treatment with boric acid and pilocarpine, on the other hand, though shortening of the upper beak and a parrot beak are sometimes found, reduction of the lower

TABLE 1

Typical symptoms of chicken embryos produced by teratogenic substances applied during the period of micromelia response

TERATOGENIC SUBSTANCE	FACIAL SKELETON	LONG BONES OF LEG	TOES	OTHER DEFECTS (usually with relatively low incidence)
Sulfanilamide	Parrot beak	Micromelia (bending of tibiotarsus)	Syndactylism	Clubbed down
Eserine	Parrot beak	Micromelia (bending of tibiotarsus)	Syndactylism	Clubbed down
Insulin	Short upper or parrot beak, rarely facial coloboma	Micromelia (maximal shortness and bending of tibiotarsus)	Syndactylism (low incidence)	Microphthalmia, buphthalmia, coloboma of upper lid
Boric acid	Short lower beak, facial coloboma, cleft palate	Micromelia (maximal shortness and bending of tarsometatarsus)	Syndactylism (shortening or lack of toes, preferentially fourth toe)	Curled toe paralysis
Pilocarpine	Depending on dose: short upper and parrot beak or short lower beak, facial coloboma, and cleft palate	Micromelia (maximal shortness and bending of tarsometatarsus)	Syndactylism (shortening or lack of first toe)	Clubbed down, microphthalmia, coloboma of upper lid
Thallium	Parrot beak	Micromelia (bending of femur and tibiotarsus)	——	Microphthalmia

beak is more typical and is frequently associated with facial coloboma and cleft palate.

In the appendicular skeleton, the long bones of the wings are much less affected by teratogenic compounds than those of the legs. Relative to body size, the legs are frequently disproportionately short (micromelia), but the extent to which the different long bones contribute to this condition varies with the treatment, as will be shown subsequently. Bending of the tibiotarsus shaft is a frequent symptom of treatment with sulfanilamide, eserine, insulin, and thallium. In thallium-treated embryos bending of the femur seems, in addition, to occur commonly (Karnofsky *et al.,* '50). After the injection of boric acid or pilocarpine, on the other hand, bending of the tarsometatarsus is most often encountered.

A varying incidence of syndactylism has been reported as a consequence of all the treatments summarized in table 1, except that with thallium. Shortening of one or more toes and complete ectrodactylism are rare features of insulin-treated embryos, but are frequently met with after injection of boric acid or pilocarpine. It is remarkable, however, that the preaxial side (first toe) suffers preferentially after pilocarpine treatment and the postaxial side (fourth toe) after boric acid.

Size of the body, as measured by weight, tends to fall below the normal range whenever one of the teratogenic substances is administered in such amounts that a high incidence of gross morphological defects is produced. No data are available for a quantitative comparison of the effects which different teratogenic compounds have on body weight, but there is little doubt that such differences exist.

In embryos and chicks of the Black Minorca breed some of the teratogenic compounds have an interesting effect on down pigmentation. Normally, the down of these chicks is solidly black with the following exceptions: the ventral body surface is predominantly covered with white down; throat and abdomen are always white, but in the pectoral region there is frequently an intermixture of black and white, resulting in a bluish appearance, or a small black area may be present, separating throat and abdomen. In addition, a few white marks are often found in the face and the alula region of the wings. We reported (Landauer, '53a) that after the injection of insulin, boric acid, or pilocarpine at 96 hours of incubation the area of black pigmentation may be greatly reduced and that in extreme cases it may be restricted to the dorsal surface of the body. The situation is diagrammatically illustrated in figure 1. It has since been found that treatment

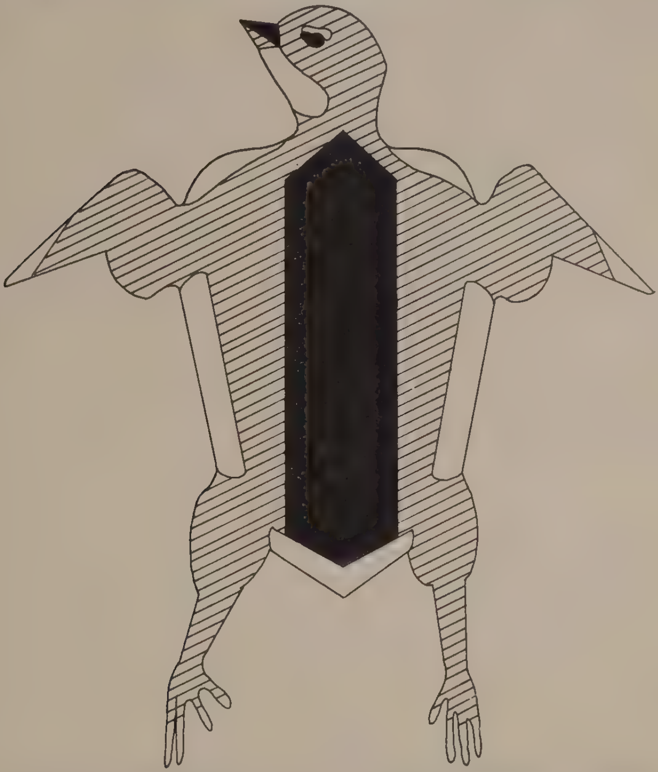

Fig. 1 Diagram illustrating the effect of various teratogenic compounds (insulin, boric acid, pilocarpine hydrochloride, and sulfanilamide), when injected at 96 hours of incubation, on down pigmentation of Black Minorca embryos and chicks. White areas are normally white (throat, abdomen, alula region of wings and small spots in face); shaded area shows maximum extension of unpigmented down in consequence of experimental interference; black area (chiefly dorsum) unaffected by teratogenic compounds.

with sulfanilamide produces similar results. Thallium, on the other hand, even if given in amounts that lead to extreme forms of micromelia in all treated embryos, has no corresponding effects on down pigmentation.

Microphthalmia was sometimes found in embryos which had been treated with insulin, pilocarpine, or thallium and buphthalmus occurs among insulin-treated chicken (and with a higher incidence in duck) embryos. Cataract occurred fairly often in insulin-treated embryos and chicks. Another malformation that has been met with not infrequently (especially after pilocarpine treatment) is a triangular coloboma of the upper lids. The buphthalmic condition may be secondary to abnormalities of skull development, but it is unlikely that the same is true for the other eye defects. Occasional symptoms, such as edema and ''clubbed'' down, probably are not a part of the specific syndromes resulting from treatment (see discussion by Ancel, '45).

DOSAGE AND EFFECT

As might be expected, the amount of teratogenic compound administered is intimately related to the resulting effect. The consequences of varying dosages may be observed on incidence as well as on type and extent of abnormality. The limiting factors are, on the one hand, a dosage too low to interfere with normal development and, on the other hand, an amount so large that most or all embryos die soon after treatment.

Observations on the incidence of rumplessness after the injection of insulin prior to incubation have shown that within a range of 0.05 to 5 units a straight-line relation obtains between dosage and effect if incidence in probits is plotted as a function of dose in logarithms. No similarly extensive observations are available for either insulin or other teratogenic compounds injected *during* incubation. The data in table 2 give some examples, however, of the relation prevailing at one particular stage (96 hours) between dosage and incidence of resulting malformations. Analogous results were

TABLE 2

Effect of dosage on incidence of abnormalities of facial and appendicular skeleton. Treatment in all instances at 96 hours. Incidence in percentage of survivors of thirteenth day of incubation. White Leghorn stock. Standard errors used throughout this report.

TREATMENT		BEAK DEFECTS	ABNORMALITIES OF LONG BONES OF LEG
Insulin (units)	2	16.9 ± 3.17	26.8 ± 3.75
	5	55.0 ± 4.97	80.0 ± 4.00
Boric acid (mg)	1	0.5 ± 0.50	0
	2.5	69.9 ± 3.69	17.6 ± 1.85
	3.5	85.3 ± 4.23	39.7 ± 5.86
Pilocarpine hydrochloride (mg)	3	0.9 ± 0.51	0.6 ± 0.42
	6	12.0 ± 1.62	29.9 ± 2.29
	12	41.8 ± 4.94	83.5 ± 3.71

TABLE 3

Incidence of syndactylism after treatment in four stages of incubation with 3, 6, and 12 mg, respectively, of pilocarpine hydrochloride. Incidence in percentage of survivors of thirteenth day. White Leghorn stock.

DOSAGE	HOURS OF INCUBATION AT TREATMENT			
	72	96	120	144
(mg)				
3	1.3 ± 1.3	16.6 ± 2.0	3.9 ± 2.2	0
6	51.1 ± 4.2	56.3 ± 2.5	83.3 ± 3.4	44.7 ± 4.5
12	68.9 ± 5.4	78.6 ± 4.1	94.4 ± 2.7	57.9 ± 4.8

TABLE 4

Means of body weight and of length of femur, tibiotarsus, and tarsometatarsus of eighteen-day chicken embryos after treatment with differing amounts of pilocarpine and insulin at 96 and 120 hours, respectively, of incubation. White Leghorn stock.

TREATMENT		BODY WEIGHT	FEMUR LENGTH	TIBIOTARSUS LENGTH	TARSO-METATARSUS LENGTH
		(g)	*(mm)*	*(mm)*	*(mm)*
Untreated		20.7 ± 0.30	17.4 ± 0.06	24.8 ± 0.14	18.6 ± 0.09
Pilocarpine (mg)	3	20.0 ± 0.26	16.7 ± 0.12	23.8 ± 0.17	17.5 ± 0.14
	6	16.6 ± 0.35	14.9 ± 0.19	20.9 ± 0.29	14.7 ± 0.29
	12	11.1 ± 0.42	12.0 ± 0.09	15.7 ± 0.44	9.9 ± 0.38
Insulin (units)	2	18.9 ± 0.46	13.6 ± 0.29	18.9 ± 0.45	15.3 ± 0.31
	4	16.9 ± 0.51	12.6 ± 0.33	17.1 ± 0.51	13.9 ± 0.42

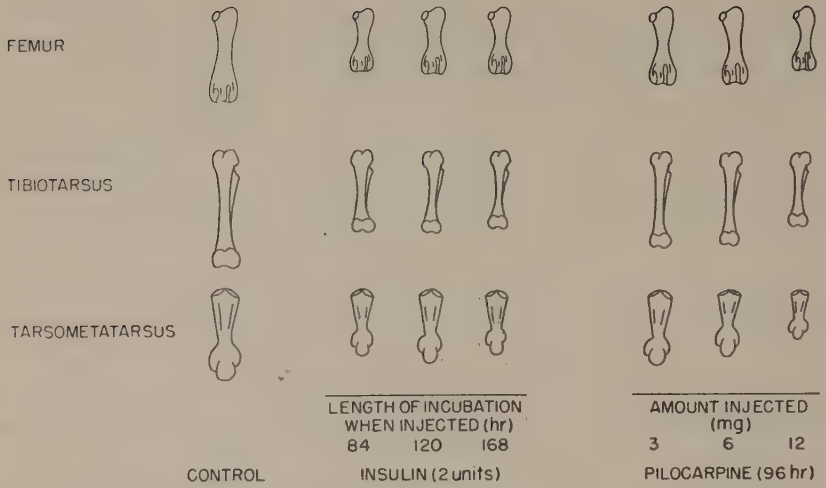

Fig. 2 Effect of insulin and pilocarpine on length of femur, tibiotarsus, and tarsometatarsus in 18-day White Leghorn embryos. Actual mean bone lengths of control and treated groups drawn to scale. The figure illustrates the results of injecting 2 units of insulin after 84, 120, and 168 hours of incubation, respectively, and those of treatment with 3, 6, and 12 mg of pilocarpine at 96 hours of incubation.

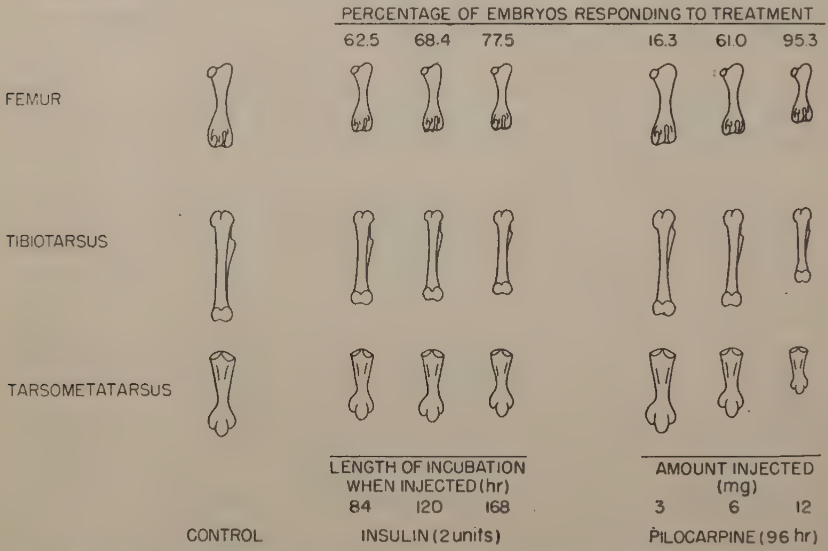

Fig. 3 The same material as in figure 2, but length of the individual bones expressed in percentage of the control mean for the same bone. The figures at the top give the percentage incidence in each group of grossly diagnosed micromelia.

found after treatment in other developmental stages. Dosage differences may also be responsible for shifts in the time of maximum incidence of a particular abnormality. Table 3 illustrates this for the production of syndactylism with varying amounts of pilocarpine.

In addition to changes in incidence, variations in dosage produce clear-cut quantitative differences in the degree of abnormality. The data in table 4 show this for body weight and length of long bones after treatment with pilocarpine (at 96 hours) and insulin (at 120 hours). Figures 2 and 3 illustrate the changes in long-bone length after varying doses of pilocarpine.

TABLE 5

Influence of three different doses of pilocarpine hydrochloride injected at 96 hours of incubation on incidence of beak defects, syndactylism, and abnormalities of first toe. White Leghorn stock.

MALFORMATION	AMOUNT OF PILOCARPINE INJECTED		
	3 mg	6 mg	12 mg
Simple beak defects	0.9 ± 0.51	7.0 ± 1.28	27.2 ± 4.44
Complex beak defects	0	5.0 ± 1.09	14.6 ± 3.53
Syndactylism	16.6 ± 2.01	56.3 ± 2.48	78.6 ± 4.11
Shortened first toe	0	7.5 ± 1.32	74.8 ± 4.34

There are differences in kind as well as in degree of malformation as a consequence of variations in dosage, although it must be kept in mind that the boundary between qualitative and quantitative differences is not always easy to discern (see the data presented in table 5). After small amounts of pilocarpine, abnormalities of the facial skeleton are very rare and confined to "simple" beak defects, i.e., shortening of the upper beak, parrot beak, or moderate degrees of shortening of the lower beak; after larger amounts of pilocarpine, on the other hand, one often observes — in addition to a much more extreme reduction of the lower beak — the presence of coloboma of the face and cleft palate. As far as the feet are concerned, small doses of pilocarpine are responsible for an appreciable incidence of syndactylism as the only abnormality, whereas after larger doses, the first toe is frequently reduced in size and, in addition, one finds reduction in length of the long bones, especially of the tarsometatarsus. These "new" malformations of face, legs, and feet, produced by high doses of pilocarpine, are presumably the result of quantitative extensions of the processes (retardation in growth of parts of the visceral skeleton and the long bones and retardation in separation and growth of foot plate) set in motion by smaller amounts.

There is no doubt, on the other hand, that qualitative distinctions exist in the manner in which various skeletal parts react to different teratogenic compounds. It is remarkable, for instance, that in experiments with insulin, eserine, and sulfanilamide syndactylism is, with rare exceptions, found only in association with moderate or severe involvement of the long bones (micromelia); but that in experiments with pilocarpine syndactylism, and nothing else, is produced by small doses, the long bones becoming involved only when larger amounts of pilocarpine are administered. These local specificities of response will be discussed in a later section.

DEVELOPMENT STAGE AND EFFECT

It has long been known, as a result of studies on many kinds of organisms, that there are definite and well-defined stages of embryonic development during which certain environmental agencies will change the fate of particular parts. These susceptible periods or susceptible stages vary from organ to organ. They frequently coincide with the first visible appearance of particular primordia, but the beginning of a sensitive stage may considerably precede the time at which a primordium can first be recognized as a morphological entity. The termination of a sensitive period, after which the course of development of a particular part is no longer susceptible to influences from without, may not be reached until after considerable growth of the part has occurred.

These facts are no cause for great surprise. Close timing of all developmental events is obviously required in order to arrive at a normally integrated and typically proportioned end result. Other features of these sensitive periods have been less well appreciated in the past. The most important of these are the following: the time of occurrence and duration of the sensitive stage for one and the same organ or part, though undoubtedly depending on intrinsic factors, may vary considerably with different external agencies (even if the same or very similar end results are produced by them) and the relative susceptibility of various parts during successive stages may be an independent function of different sources of experimental interference.

Figure 4 illustrates how the incidence of malformations of the visceral and appendicular skeleton varies with time of injection following treatment with insulin. Incidence varies with dose, as has been discussed previously, but for one and the same teratogenic compound and the same target area the shapes of the response curves are similar, except that with increasing doses the period of response may begin earlier and last longer.

A quantitative comparison of the effect on long bone length produced by 2 units of insulin was made on three groups of 18-day embryos which had been treated at 84, 120, and 168 hours of incubation, respectively (Landauer, '53e). The results are shown in table 6 and figures 2 and 3. In contradistinction to the striking dosage-effect relations discussed previously, it will be noted that the degree of shortening of the long bones remains nearly constant after treatment between the middle of the fourth and the end of the seventh day of development. Only the tibiotarsus shows a slight (statistically nonsignificant) trend toward greater reduction in length with increasing age at time of treatment. The realness of this trend was substantiated by the analysis of relative tibiotarsus length shown in table 7. The values for relative tibiotarsus length, as expressed in percentage of combined length of the three long bones, were for each experimental group divided into three subgroups according to ranges of combined long-bone length. It can be seen that mean relative tibiotarsus length decreased at the two higher range levels from the early to the late stage of treatment. The differences are small, but significant. Yet, on the whole, it is evident that with one and the same dosage the absolute and relative changes

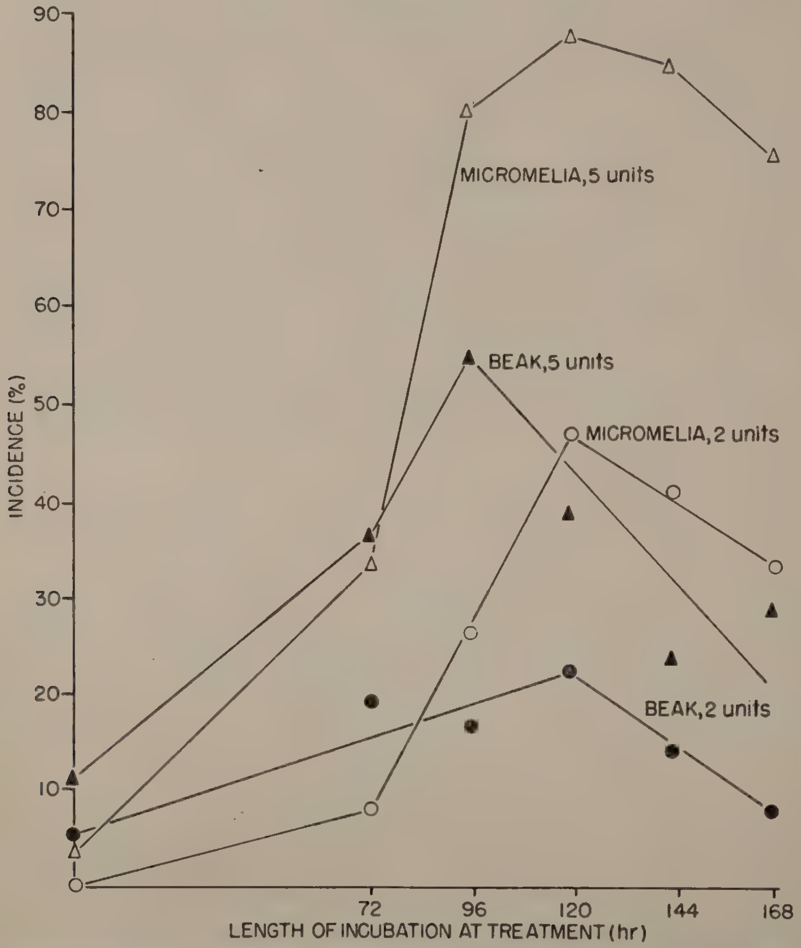

Fig. 4 The incidence of beak defects (all types) and micromelia among White Leghorn embryos and chicks treated between 0 and 168 hours of incubation with 2 and 5 units of insulin, respectively. Data based on survivors of thirteenth day of incubation.

in long-bone length, produced by a teratogenic substance, remain nearly constant during the responsive period. This relative lack of quantitative changes with time is in sharp contrast to the rise in incidence of malformations occurring (with a constant dose of insulin) between 84 and 168 hours (and amounting to 62.5, 68.4, and 77.5%, respectively).

TABLE 6

Means of greatest length of femur, tibiotarsus, and tarsometatarsus of eighteen-day chicken embryos, treated at 84, 120, and 168 hours, respectively, with 2 units of insulin. White Leghorn stock.

TIME OF TREATMENT	FEMUR LENGTH	TIBIOTARSUS LENGTH	TARSOMETATARSUS LENGTH
(hr)	*(mm)*	*(mm)*	*(mm)*
Untreated	17.4 ± 0.06	24.8 ± 0.14	18.6 ± 0.09
84	13.6 ± 0.36	19.1 ± 0.59	14.6 ± 0.44
120	13.6 ± 0.29	18.9 ± 0.45	15.3 ± 0.31
168	13.3 ± 0.78	17.6 ± 0.61	14.2 ± 0.44

TABLE 7

Mean length of tibiotarsus in percentage of combined long-bone length and in groups according to ranges of combined long-bone length. Data from 18-day White Leghorn embryos treated with 2 units of insulin at 84, 120, and 168 hours, respectively. Those embryos were omitted from all groups whose combined long-bone length exceeded the control mean less 2 standard deviations.

Insulin 2 units, 84 hr	Range of combined length (mm)	17.4 – 29.0	29.1 – 41.0	41.1 – 53.4
	Mean relative tibiotarsus length (%)	37.7 ± 1.92	39.8 ± 0.32	40.6 ± 0.22
Insulin 2 units, 120 hr	Range of combined length (mm)	22.7 – 33.2	33.3 – 43.7	43.8 – 53.8
	Mean relative tibiotarsus length (%)	38.2 ± 0.95	38.4 ± 0.44	39.6 ± 0.19
Insulin 2 units, 168 hr	Range of combined length (mm)	21.8 – 32.0	32.1 – 43.0	43.1 – 53.7
	Mean relative tibiotarsus length (%)	37.4 ± 1.09	38.3 ± 0.21	38.8 ± 0.58
Difference between means of 84- and 168-hr stages			1.5 ± 0.38	1.8 ± 0.62

Micromelia is presumably the result of disturbances in growth of the long bones. It seems likely that the abnormalities of the visceral skeleton are likewise the result of a failure of the constituent cartilaginous primordia to grow normally. In particular circumstances, disharmonies and dislocations of growth result in gross malformations such as

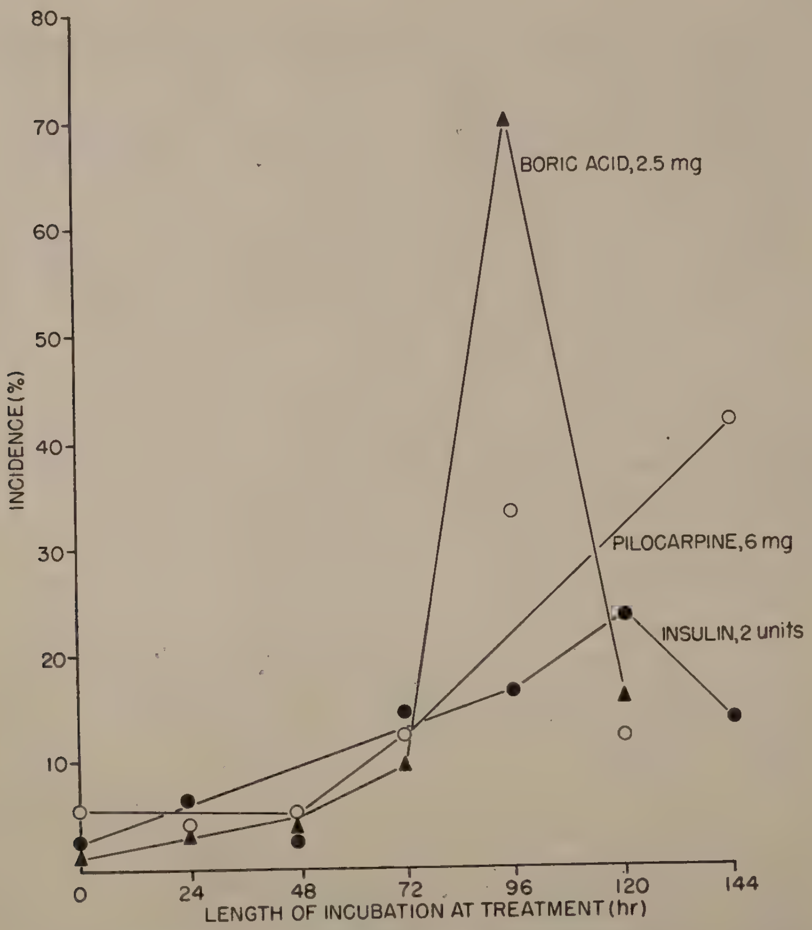

Fig. 5 Incidence of beak defects (all types) among White Leghorn embryos and chicks treated between 0 and 144 hours of incubation with 2 units of insulin, 2.5 mg of boric acid, and 6 mg of pilocarpine, respectively. Data for survivors of thirteenth day of incubation.

syndactylism, bending of tibiotarsus or tarsometatarsus, and cleft palate. The period of development during which these secondary defects can be produced (by insulin) with any degree of frequency is much more limited than is true for the primary growth retardations.

A comparison of the effects of different teratogenic substances shows that for the same organ or part the time of onset, duration, and maximum of susceptibility may vary con-

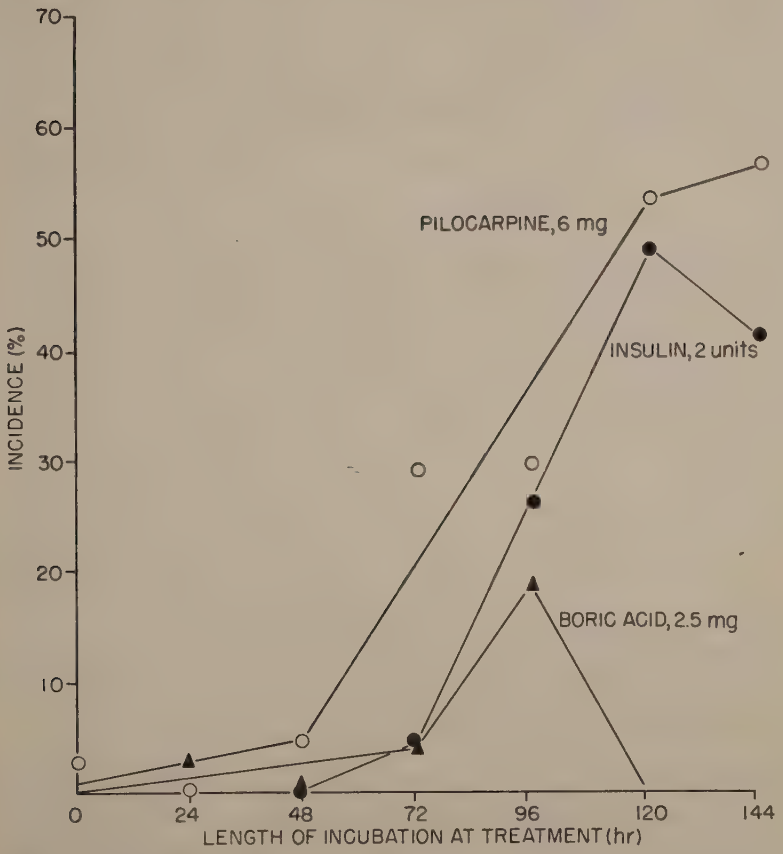

Fig. 6 Incidence of long-bone defects (legs) among White Leghorn embryos and chicks treated between 0 and 144 hours of incubation with 2 units of insulin, 2.5 mg of boric acid, and 6 mg of pilocarpine, respectively. Data for survivors of thirteenth day of incubation. Diagnosis based on gross observation.

siderably from one compound to another. This problem is, of course, complicated by the influence of dosage and other factors on these features of the susceptible periods, as mentioned in an earlier section. Such differences in specificity of local response are illustrated in figures 5 and 6. At the dosage levels chosen for this comparison, incidence of malformations of beak and of extremities differs little in early stages after treatment with insulin, boric acid, or pilocarpine. After boric acid treatment the incidence of beak defects rises to an earlier and much higher maximum than after treatment with the other two compounds and the incidence also drops much

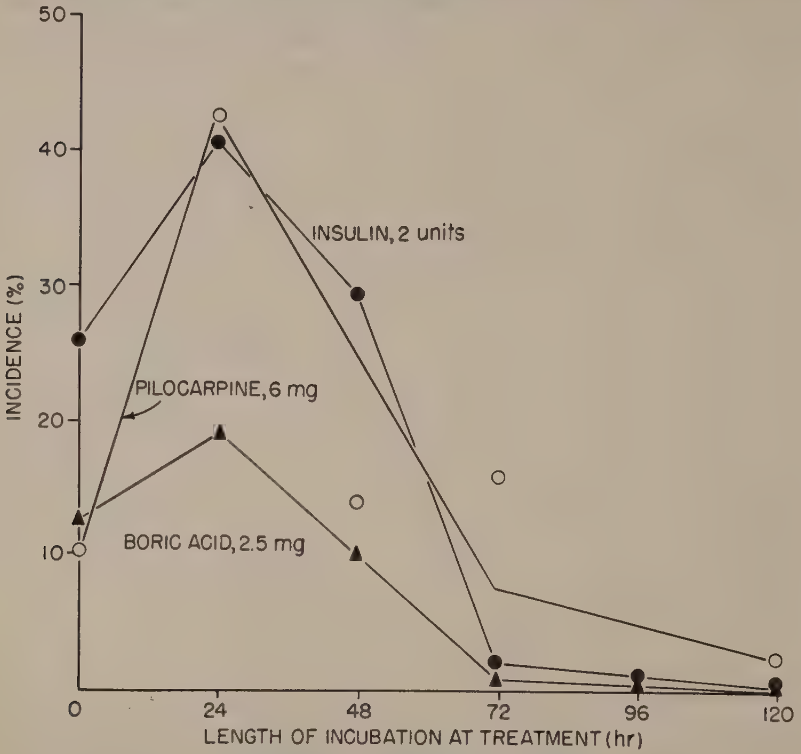

Fig. 7 Incidence of rumplessness among White Leghorn embryos and chicks treated between 0 and 120 hours of incubation with 2 units of insulin, 2.5 mg of boric acid, and 6 mg of pilocarpine, respectively. Data for survivors of thirteenth day of incubation.

more precipitately beyond the stage of greatest effectiveness. In the extremities, on the other hand, boric acid produces a much lower incidence of abnormalities than either insulin or pilocarpine, but — as in the case of beak defects — the period of response is again more limited in time. The effects of insulin and pilocarpine differ less strikingly (at the particular dosage levels), but it is remarkable that with pilocarpine treatment the incidence of beak and appendicular defects rises to the last stage tested (144 hours). For comparison, figure 7 shows the incidence of rumplessness following injection of the same three compounds and in the same amounts. The general trend is similar in all instances, but boric acid is less damaging to the tail primordium than are the other two substances.

GENOTYPE AND EFFECT

A good deal of evidence is available for the conclusion that the genotypic constitution of an animal is of great importance for the outcome of experiments with teratogenic substances. It is a common experience that even in tests with compounds (and in concentrations) which produce severe malformations, some embryos escape all harm or may be only slightly affected. Some authors have taken this as proof for the existence of uncontrolled environmental factors. This may, indeed, be an important source of variance, but it certainly does not account for all exceptions.

Intrabreed variance. In one of our early experiments with insulin we found that a stock of White Leghorn fowl showed a significant degree of heterogeneity between mothers in regard to the incidence of rumplessness. With selection based on these data, it was possible to establish at once two lines which for several generations gave consistently different results when insulin was injected in the earliest stages of incubation (2–4 hours). The eggs of these high- and low-response lines did not yield significant differences when treatment occurred prior to incubation. In unselected stock, the incidence of rumplessness after insulin treatment gradually rose during incubation until a maximum was reached after 24–30 hours. It seems likely, therefore, that the high- and low-response lines differed in the rate at which development got under way and at which, consequently, levels of differing susceptibility were attained (Landauer and Bliss, '46).

In another experiment in which eggs from a mating of Black Minorca fowl had been treated with 2.5 mg of boric acid after 96 hours of incubation the resulting data gave clear evidence of heterogeneity of the mothers (Landauer, '53c). The eggs had been produced by eighteen hens. These progenies were analyzed for freedom from all malformations ($\chi^2 = 29.21$, df $= 17$, P about 0.025), occurrence of beak defects ($\chi^2 = 28.71$, P about 0.025), and presence of tarsometatarsus abnormalities ($\chi^2 = 38.59$, $P < 0.01$). There is little doubt that in all three respects significant heterogeneity had occurred. Since some of the hens had produced only small progenies, the calculations were repeated for the ten progenies with ten or more embryos each. The results thus obtained indicate that the response differences between the progenies of these ten Minorca hens were highly significant (with probabilities of < 0.01 in all three instances). Similarly, we found that a group of White Minorca hens showed highly significant heterogeneity in the occurrence of tarsometatarsus defects as a response to boric acid treatment. Such examples could be multiplied. In some instances of intrabreed (and also interbreed) variance in response we are unquestionably dealing with the result of maternal effects.

Interbreed variance. The existence of breed differences in response to insulin is as well established as the reality of intrabreed heterogeneity. It is well known, of course, that the term "breed" as applied to fowl is loose and genetically poorly defined. It indicates, at any rate, a more distant relationship than would ordinarily be found within breeds and, in conformity with this fact, interbreed differences in response to teratogenic compounds tend to be more striking and are demonstrable with greater ease. We shall here con-fine ourselves to one particularly instructive example. The incidence of facial defects as well as of malformations of the extremities after injection of boric acid is much greater among Silver Gray Dorking than among White Leghorn embryos, as can be seen from the data in table 8. Yet, the two breeds do not differ significantly in their responses to insulin after treatment during the same stage. In evaluating these results it must be kept in mind that the malformations of facial and appendicular skeleton are of a somewhat different nature according to whether they are produced by boric acid or insulin.

Maternal effects. In some instances it was possible to demonstrate that the degree of response to a teratogenic compound was influenced or determined by physiological traits peculiar to the mother. It could be shown for instance (Landauer, '48a) that, following injection of 2 units of insulin prior to incubation, the incidence of rumplessness in reciprocal crosses of White Leghorn and Jungle fowl coincided almost exactly with that found in the maternal stock (Leghorn 25.7 ± 3.0%, Jungle 9.3 ± 2.9%). No significant response differences were found in these crosses when insulin was injected at the 120-hour stage. It may well be that in these reciprocal crosses, and also in the previously mentioned White Leghorn lines selected for high and low response to insulin, the dissimilarities in response were brought about by maternally determined differences in rate of early development. This would account for similar results not being observed after insulin treatment in later stages.

There is also evidence, however, that maternal factors not concerned with early developmental rate may influence the result of experiments with teratogenic compounds. One such factor is related to plumage pigmentation, another to seasonal trends.

Effects of plumage pigmentation. In reciprocal matings of Black and White Minorca fowl in which (because of heterozygosity of the white parents) segregation in regard to plumage color occurred among the progenies, it was found

TABLE 8

Differences between White Leghorn and Silver Gray Dorking embryos in response to the injection of boric acid and insulin after 96 hours of incubation

TREATMENT	FACIAL DEFECTS			ABNORMALITIES OF EXTREMITIES		
	White Leghorn	Silver Gray Dorking	Difference	White Leghorn	Silver Gray Dorking	Difference
Boric acid 2.5 mg	60.3 ± 3.42	93.7 ± 1.57	33.4 ± 3.76	23.4 ± 2.97	50.8 ± 3.23	27.6 ± 4.39
Insulin 2 units	16.1 ± 3.35	29.2 ± 5.64	13.1 ± 6.56	29.7 ± 4.18	18.5 ± 4.82	11.2 ± 6.38

TABLE 9

Data comparing the response to boric acid of phenotypically white embryos segregating in reciprocal crosses of Black and White Minorca fowl. Incidence of curled-toe paralysis in percentage of hatched chicks; incidence of other traits in percentage of survivors of thirteenth day of incubation. The size of the samples on which the percentages are based is shown in parentheses.

MALFORMATION	WHITE EMBRYOS AND CHICKS FROM		SIGNIFICANCE OF DIFFERENCE	
	19 white mothers	20 black mothers	χ^2	P
Curled-toe paralysis	6.7 (90)	48.5 (33)	28.77	< 0.0001
Free of malformations	40.7 (268)	28.9 (204)	6.97	< 0.01
Complex beak defects	17.2 (268)	38.2 (204)	26.54	< 0.0001
Toe defects	3.4 (268)	11.5 (204)	9.28	< 0.01

that after treatment with boric acid a greater number of white than black embryos and chicks were free of induced malformations ($\chi^2 = 12.07$, $P < 0.001$). Of more immediate interest for the present discussion is the observation, however, that the maternal plumage color also influenced the fate of boric acid-treated embryos indirectly. In order to exclude the complicating factor of embryonic down pigmentation, data are here presented for only the white embryos and chicks from the two reciprocal Minorca matings (table 9). These figures show clearly that in the progenies of black mothers, as compared with those of white mothers, the chances of embryos and chicks of escaping damage from boric acid injection were much smaller and the incidence of curled-toe paralysis, complex beak defects (i.e., beak defects complicated by facial coloboma and cleft palate), and abnormalities of the toes were much greater. The causes of these response differences remain unknown, but are probably related to egg composition, as influenced by maternal plumage pigmentation.

Seasonal trends. In experiments with insulin and boric acid it was observed repeatedly that incidence and degree of the induced malformations (penetrance and expressivity in genetical terminology) change with advance of the season in such a way that, with rising mean temperatures of the external environment, a lessening of the teratogenic effectiveness of these compounds tends to take place. The occurrence and extent of such seasonal trends appear, however, to depend on the genetic constitution of the stock in question. This is illustrated by the data in table 10 from boric acid experiments with Black and White Minorca fowl. In Black Minorca embryos the incidence of tarsometatarsus defects declined sharply from early to late spring, but in White Minorca embryos it remained about the same, resulting in a highly significant breed difference in seasonal trend ($\chi^2 = 27.514$, $P < 0.0001$). Black Minorca embryos also showed a precipitous seasonal drop in frequency of complex beak defects, whereas the corresponding figures for White Minorca em-

TABLE 10

Incidence during early and late spring of complex beak and tarsometatarsus defects among embryos from Black and White Minorca fowl after treatment with 2.5 mg of boric acid at 96 hours of incubation

STOCK	COMPLEX BEAK DEFECTS			TARSOMETATARSUS DEFECTS		
	Early spring	Late spring	Significance of difference	Early spring	Late spring	Significance of difference
	(%)	(%)		(%)	(%)	
Black Minorca	57.6 ± 3.22	32.1 ± 2.82	$\chi^2 = 33.407$ $P < 0.0001$	45.3 ± 3.24	16.1 ± 2.21	$\chi^2 = 52.083$ $P < 0.0001$
White Minorca	63.4 ± 4.20	47.7 ± 3.39	$\chi^2 = 8.317$ $P < 0.01$	22.4 ± 3.63	24.8 ± 2.92	$\chi^2 = 0.265$ $P = \sim 0.62$

bryos indicate that a much less extreme, though significant, decline had occurred among them. These differences between the two stocks in the seasonal trend in incidence of complex beak defects are also highly significant ($\chi^2 = 10.164$, $P = 0.001$). The seasonal trends of response to teratogenic compounds, observed in our material, may be superimposed upon naturally occurring events of a similar kind. That such natural trends exist has been demonstrated for the amino acid composition of eggs and is indicated by the well-established seasonal gradients in the incidence of sporadic microphthalmia and chondrodystrophy.

The effect of genetic "modifiers." Attention has previously been called to the fact that modifiers of certain mutant conditions may also modify the effectiveness of teratogenic compounds. Observations of this kind were made in the course of insulin experiments with embryos from two stocks carrying lethals. One of these is our stock of Creeper fowl in which unanalyzed genes enhancing growth in length of the long bones (and survival of the Creeper homozygotes) are known to exist. The other is a stock in which conscious and successful selection against expression of the so-called "short upper beak" lethal, which also reduces long-bone length, had been practiced. Both stocks were notably refractory to the action of insulin on long bones and even to its effects on development of the tail (Landauer, '47a).

Action of teratogenic agents paralleling that of modifying genes was encountered in experiments with polydactylous fowl (Landauer, '48b). The variants of the hereditary five-toed condition of fowl occur in the presence of multiple modifiers. The incidence of one or another of these aberrant forms can be raised by selection. These variants include complete suppression of polydactylism, heterodactylism, polyphalangism, and the existence of more than five toes on each foot. Polyphalangism, i.e., the presence of a lengthened first toe in place of a duplicated one, can most readily be isolated by selection. It was also shown that, after insulin treatment of genetically polydactylous embryos, polyphalangism was the most frequent experimental modification. Additional proof for the same conclusion was obtained from experiments in which eggs of Dorking fowl, homozygous for polydactylism, received an injection of 2.5 mg of boric acid at 96 hours of incubation. The results are shown in table 11, giving percentage incidence of the different forms of expression. Whatever the nature of genetic and experimental modifications of the polydactylous condition may be, it seems reasonable to assume that in the two situations the shifts in expression are brought about in similar ways.

TABLE 11

Effect of boric acid on toe formation in Dorking chicks

MALFORMATIONS NO. OF CHICKS →	UNTREATED CONTROLS 262	BORIC ACID TREATED 176
Typical bilateral polydactylism	76.0 ± 2.6	5.1 ± 1.7
Atypical bilateral polydactylism	2.3 ± 0.9	2.8 ± 1.2
Heterodactylism	16.4 ± 2.3	3.4 ± 1.4
Nonpolydactylous	5.3 ± 1.4	5.7 ± 1.7
Polyphalangism	0	83.0 ± 2.8

SELECTIVE AFFINITIES OF TERATOGENIC COMPOUNDS

The bearing which developmental stage at the time of treatment, dosage, genotype of the treated embryos, and seasonal factors have on the morphological effect produced by the various teratogenic compounds is, essentially, of a quantitative nature. If, on the other hand, a comparison is made of the specific manner in which the different teratogenic substances interfere with normal development, qualitative dissimilarities are found in their affinities to particular skeletal parts. Before giving examples illustrating this important fact, it must be recalled that the teratogenic compounds under discussion are similar in that, if given at the proper stage and in sufficient amount, they will lead to defects of the facial and appendicular skeleton.

Two groups of teratogenic substances can be distinguished according to their effects on the facial skeleton and the long bones of the leg, viz., insulin, sulfanilamide, and eserine sulfate on the one hand and, on the other, boric acid and pilocarpine hydrochloride. The micromelia produced by insulin, sulfanilamide, and eserine is characterized by a nearly proportionate reduction in length of the three long bones, although the tibiotarsus always suffers to a somewhat greater extent than the femur and tarsometatarsus. The micromelia induced by boric acid and pilocarpine features, in contradistinction, much the most severe effect on length of the tarsometatarsus. Figures 2 and 3 illustrate these relations for insulin and pilocarpine.

The feet may, in the presence of extreme forms of micromelia, be reduced in size after treatment with insulin, sulfanilamide, and eserine, but selective shortening or complete suppression of toes has been observed in only rare instances. On the other hand, micro- and ectrodactyly are common and distinguishing consequences of experiments in which boric acid or pilocarpine were used. This is in conformance with the affinity of these substances for the more distal skeletal parts (Landauer, '52, '53d). It is similarly characteristic that syndactylism occurs as the first (and after small doses the only) symptom of pilocarpine treatment, but that in experiments with insulin, sulfanilamide, and eserine it is, as a rule, observed only in association with the more extreme forms of micromelia.

A shortening of the upper beak or the parrot beak condition are the usual effects of treatment with insulin, sulfanilamide, or eserine. Facial clefts were observed only exceptionally in insulin-treated embryos and never after the injection of sulfanilamide or eserine. None of the three compounds led to a pronounced shortening of the mandible. In contrast, marked and frequently extreme shortening of the mandible was a typical trait of embryos that had been treated with boric acid or pilocarpine, and this condition was often associated with facial coloboma and cleft palate.

It is evident from these observations that the two groups of teratogenic compounds produce their effects in somewhat different ways and this conclusion is supported by the fact, to be discussed more fully hereafter, that the effects of insulin, sulfanilamide, and eserine can be completely forestalled by administering supplementary nicotinamide, but that in combination with boric acid and pilocarpine nicotinamide gives only partial protection. One finds, however, distinguishing features also within each of these two groups of teratogenic substances. An interesting example for this is furnished by the dissimilar reaction of toe development to treatment with boric acid or pilocarpine. In pilocarpine-treated embryos the first toe is frequently greatly reduced in size, but other toes are not affected, except for the syndactylism between third and fourth toes. Boric acid treatment, on the other hand, produces a graded series of size reduction and complete loss of toes, and in this series the fourth toe is most readily involved, the first toe follows next, the second and third toes being least frequently reduced in size or entirely lacking. This sequence of toe involvement remains the same over the whole period during which pilocarpine and boric acid interfere with foot development.

The micromelia produced by thallium which, for want of detailed information, has not been included in the foregoing discussion, seems to show at least one feature that is not found in the other syndromes, viz., bending of the femur shaft.

EMBRYO METABOLISM AND TERATOGENIC COMPOUNDS

Observations relating to the biochemical mechanisms by which teratogenic substances produce their effects during development of the chicken embryo will now be reviewed briefly. Our scanty information on this subject comes chiefly from experiments with various supplements, a few assays, tests with inhibitors of anaerobic glycolysis, blood sugar determinations, and, of course, deductions from related facts found in the literature.

During the two principal periods in which insulin interferes with normal development embryos can be protected against the teratogenic action of the hormone by providing adequate amounts of certain supplements (Landauer, '48b, '49; Landauer and Rhodes, '52). For effective protection embryos require different supplements during the two periods. In early stages, during which rumplessness is the typical consequence of insulin treatment, pyruvic acid (or sodium pyruvate) gives the best known protection. The same holds for rumplessness occurring as a consequence of sodium cacodylate administration. Lactic, citric, and α-ketoglutaric acids — in the sequence named — have, during the same stages, decreasing value as protective supplements. With the beginning of the second major susceptible period, during which insulin treatment tends to cause micromelia and beak defects, all of these compounds cease to be valuable as supplements and are rendered toxic. Nicotinamide, which has considerable toxicity in earlier stages, becomes the supplement with outstanding protective value. These observations clearly indicate that the action of insulin by which rumplessness is produced during early stages and the long bone-beak syndrome later, must be mediated over somewhat different pathways, corresponding to changing metabolic functions of the embryo.

Whereas nicotinamide protects embryos completely against insulin damage to the facial skeleton and the long bones (at least at the level of 2 units of insulin supplemented by 5 mg of nicotinamide), other supplements were found to have a differential effect. Thus citric acid, glucose-1-phosphate, and oxaloacetic acid significantly reduce the incidence of beak defects after insulin treatment, but they apparently do nothing to lessen damage to the long bones. Conversely, lactic acid lowers the incidence of insulin-induced micromelia without furnishing protection to the facial skeleton. Zwilling and DeBell ('50) observed similarly in their experiments with sulfanilamide that the occurrence of micromelia could be completely prevented with supplementary nicotinamide, but that damage to the beak persisted. After eserine sulfate, on the other hand, nicotinamide gave, as in conjunction with insulin, complete protection against appearance of the whole syndrome. Finally, in combination with boric acid treatment supplementary nicotinamide reduced the incidence of facial abnormalities, but had little, if any, beneficial value in regard to micromelia.

With other supplementary compounds differential potentiation of the insulin effects can be produced. Among such compounds are some, e.g., sodium succinate and 3-hydroxy-anthranilic acid, which are quite harmless when injected by themselves and others, e.g., adrenal cortex extract (cortisone acetate), which retard general body growth but without producing teratological results (Landauer, '47b). The data in table 12 for incidence of malformations illustrate, as an example, the potentiating effects which 3-hydroxy-anthranilic acid had on treatment with 2 units of insulin injected after 96 hours of incubation.

TABLE 12

The extent of potentiation, according to body parts, produced by 3-hydroxy-anthranilic acid on insulin-induced malformations

	PERCENTAGE INCIDENCE OF		
	Micromelia	Beak defects	Syndactylism
Insulin alone	60.0 ± 2.38	26.1 ± 2.13	4.5 ± 1.01
Insulin + 4 mg of 3-hydroxy-anthranilic acid	89.2 ± 3.86	69.2 ± 3.75	36.9 ± 6.00
Potentiation (%)	48.7	165.1	720.0

Experiments with metabolic supplements can give information about the speed with which teratogenic compounds produce irreversible changes of development. In this way it was found that nicotinamide had lost its protective value when it was injected as little as 6 hours subsequent to insulin treatment at 120 hours. Similarly, pyruvic acid supplementation was of little avail when its injection was delayed for 3 hours beyond insulin treatment at 24 hours. It must be concluded from these observations that the damage done by insulin occurs within a very short time after its injection.

It is more difficult to interpret situations in which supplementation remains effective over an extended period. Zwilling and DeBell have shown that micromelia due to sulfanilamide injection at 5 days could still be completely forestalled by giving nicotinamide 48 hours later; the same was true when sulfanilamide was given after 48 hours and nicotinamide after 120 hours of incubation. Since the observations of Zwilling and DeBell indicate that with sulfanilamide treatment the maximum micromelia incidence occurs at the 48-hour stage, it seems most reasonable to assume that in combination with this teratogenic compound nicotinamide, besides serving as a protective supplement, may also (following delayed injection) act as a sufficient stimulus to overcome damage that had already occurred.

The micromelia due to injection of insulin, sulfanilamide, and eserine is (within certain dosage limits and with proper amounts of supplement) entirely preventable by providing nicotinamide. Full protection of the facial skeleton against damage from insulin and eserine requires greater care in dosage of the supplement, but can also be accomplished. In contradistinction, and parallel to differences in morphological symptoms, such effectiveness of nicotinamide is lacking if used as a supplement to injection of boric acid and pilocarpine. The incidence of malformations is lowered by nicotinamide supplementation, but is far from abolished. It was impressive from the start that defects due to boric acid treatment bear a striking resemblance to symptoms of embryonic riboflavin deficiency. Curled-toe paralysis, a condition which had previously been known only from chicks produced on riboflavin-deficient maternal diets, is common among chicks from boric acid experiments. Shortening of the lower beak and disproportionate shortening of the tarsometatarsus are likewise features characteristic of both conditions. The suspicion of relatedness in origin was strengthened by the results of assaying the livers of boric acid-treated and control embryos for riboflavin. In the treated group the livers were grossly deficient in mean riboflavin content. We assume, as a consequence, that in our experiments some riboflavin-containing enzyme, or enzymes, has been made unavailable to the embryo by complex formation of riboflavin with boric acid. Support for this interpretation was obtained by the observation that any substances known to form complexes with boric acid, such as ribose, sorbitol, and riboflavin itself, will, when added to boric acid prior to injection (or if injected simultaneously), render the latter quantitatively nonteratogenic (Landauer, '53b). We also have evidence that elementary boron is the complexing and teratogenic agent of boric acid and that aluminum salts (sodium aluminate) produce somewhat similar, if much weaker, effects.

In another series of experiments — testing the effect of inhibitors of anaerobic glycolysis on embryonic development — it was found that such compounds, even when used in toxic amounts, did not interfere with normal development of the facial and appendicular skeleton. When, however, these same substances were injected in early stages of development (0 and 24 hours) some of them, especially phlorizin and monoiodoacetic acid, led to a rather high incidence of rumplessness. This is not only additional proof that during our early and late stages malformations are brought about via somewhat different pathways, but is at the same time another demonstration that these pathways are concerned with carbohydrate utilization.

The important manner in which steps of carbohydrate metabolism are involved in the origin of our experimentally induced malformations was further elucidated by the studies of Zwilling ('48 to '51) on blood sugar. The principal results of his work were, very briefly, as follows: The injection of insulin into the yolk sac (at any time between 30 hours and 5 days of incubation) led to a pronounced hypoglycemia. Treatment with sulfanilamide had no effect on the blood sugar level. Treatment with adrenal cortex extract (which causes dwarfing of the embryo, but without gross morphological defects) was followed by a moderate hyperglycemia. Simultaneous injection of insulin and cortin produced a potentiation (in incidence and degree) of the insulin-induced malformations and an exaggerated drop of the blood sugar level. All changes in glycemia were dosage dependent. The blood sugar concentration of insulin-treated embryos tended to return to normal at about the fourteenth day, presumably coincident with beginning functioning of the pancreas and other endocrine glands, but this recovery was generally delayed after simultaneous treatment with insulin and cortin. In addition to the dosage-effect relation, the course of the hypoglycemic reaction varied also between embryos in such a way that, after a constant dose, the extent to which the blood sugar level was depressed and the time and speed of recovery paralleled the degree of skeletal abnormality.

It was further shown by Zwilling that after insulin treatment the yolk sac membrane is enriched in glycogen and total carbohydrate content, whereas the embryo shows a lowering in all carbohydrate fractions. There is no doubt that insulin inhibits glycogenolysis in the yolk sac membrane. It is known, from the experiments with sulfanilamide, however, that malformations very similar to those due to insulin can be brought about in the absence of hypoglycemia; it is also known that protective supplementation of insulin treatment (with pyruvic acid in early stages, with nicotinamide later), in order to be effective, must take place within a brief period after the injection of insulin. Hence, it seems justified to conclude that the teratogenic substances dealt with here have a direct effect on the embryo, in addition to their interference with the ''transitory liver'' functions of the yolk sac membrane.

DISCUSSION

In more general considerations, the over-all similarity of the syndromes which have been discussed must be emphasized anew. In all of them were found defects of the facial and appendicular skeleton associated with each other and, in black chicks, with abnormalities of down pigmentation. Differences in detail of expression will be reviewed later. A great variety of chemical compounds, besides those here discussed, have been used during the same developmental stages: they either had no teratogenic effects or they produced abnormalities related to these same syndromes.[2] Moreover, syndromes of the same general nature, viz., associated malformations of the facial and appendicular skeleton, have become known from two other sources of investigation (for review see Landauer, '51b). In experiments relating to the developmental effects of vitamin B deficiencies, it was found that, in eggs produced on maternal diets deficient in riboflavin or biotin, many embryos showed a complex of similar morphological defects. Symptoms of a grossly and probably biochemically related nature arise in the presence of manganese deficiency. Again, out of seventeen genetic lethal conditions now known in fowl, no fewer than eight exhibit associated abnormalities of beak and extremities. That all these facts are the product of coincidence, surely no one will believe. It may, on the contrary, be safely assumed that this widespread similarity of syndromes is an expression of physiological relatedness, though not necessarily identity, of the causative pathways. By the same token, it is likely that the variations in detail between syndromes indicate specificity of response to particular conditions.

Great interest has always been aroused by the so-called "critical periods" during development which we prefer to designate as sensitive or susceptible stages. Our evidence demonstrates that the limits of these periods, beginning as well as end, depend to a certain extent on dosage. The same is presumably true for other agents, e.g., X rays. With greater amounts of a teratogenic compound it is frequently possible to obtain malformations which with smaller doses make their appearance only after treatment in later stages. It is not clear as yet if this is so because earlier stages are actually damaged or because sufficient amounts of undetoxified material remain until the actual inception of the sensitive period. The terminal dosage-stage extension is less impressive, but certainly due to a direct dosage effect. With a constant dose the variations of effect in the course of the sensitive period are of a quantitative nature. It is remarkable, however, that the maxima of incidence and of severity of effect need not coincide. In the experiments with insulin (where a susceptible period of long duration is involved) the incidence of responding embryos continued to rise throughout the stages tested. This, presumably, means that with advancing age more and more embryos had reached the threshold of beginning response and that few, if any, had arrived at a stage beyond which no response occurred. During the same time, however, the effect of insulin, as measured by long-bone length, did not change materially. The explanation of this situation may lie in the fact that the developmental gain after treatment in later stages, as expressed by a lesser compound-interest accumulation of retarded growth, was quantitatively offset by a growing scarcity of certain critical compounds and, consequently, a more drastic effect if their availability was interfered with.

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[2] Cortisone, even if injected in early stages, does not seem to interfere with embryo growth until after the eighth day of incubation (Karnofsky *et al.*, '51). Cavallero *et al.* ('52) have published evidence implicating interference with protein synthesis.

In our experiments the different parts of the syndromes are not inseparable entities. Wherever, in fact, there was opportunity to study the sequence of events in sufficient detail, it was found that the maximum incidence of facial defects tends to occur somewhat earlier than that of malformations of the appendicular skeleton.

The general features of the sensitive periods vary greatly from one teratogenic compound to another. There is wide variation in the duration of sensitiveness to a particular substance and in the maxima of teratological response, as has been shown; and during different periods the responses may vary independently. Indeed, as was first emphasized by Ancel, some substances are effective at only one of the susceptible stages. These facts can be understood only on the basis of specific interactions between the teratogenic chemicals and the affected parts of the embryo.

The dosage-effect relations are of a purely quantitative nature which, in the case of insulin, could be shown to follow a straight line. This, of course, should not obscure the possible interdependence of quantity and quality. The gradations leading from brachydactylism to ectrodactylism are a pertinent example.

Since we knew of the great powers of adaptability to alien forces of the environment, possessed by most organisms, and also of the abundant resources of variability and selection represented by the so-called genetic modifiers, it is not surprising to find that response differences of a genetic nature exist within single breeds as well as between different breeds of fowl or between different orders of birds (Landauer '51a). Observations of a similar kind have been made in mice (Fraser and Fainstat, '51; Fraser *et al.,* This Supplement), and the resistance to toxic, if not teratogenic, substances has long been a subject of genetic studies. The hereditary differences in response, as observed in our material and also by Ancel ('50), are expressed in quantitative variations, especially of the incidence of malformations, but in some cases also of the severity of expression.

Real interest, I believe, attaches to these genetic variants in response only insofar as they help to give (1) an insight into the mechanisms influencing and guiding response rates or (2) as they have a bearing on the relations between phenocopies and mutants. Under the first heading, maternal influences were seen to play an important role in determining the extent of response to teratogenic agencies. Experiments with insulin showed that the incidence of abnormalities during the rumplessness-inducing stages can be altered by selection and that the incidence differences are based on maternally determined variations in the initial rates of development. These dissimilarities of response are not carried over into later susceptible periods. In another example — that of differences in response to boric acid treatment at 96 hours found in reciprocal crosses of white and black fowl — it seems very likely that the causes must be sought in dissimilarities of egg composition ascribable to disparities of maternal physiology. Under the second heading there is the intriguing observation that stocks which carry genes producing a syndrome of facial and appendicular malformations when homozygous and which also carry plus modifiers of these syndromes are highly resistant to insulin treatment, and this during both the early and late sensitive stages.

Several independent sources of evidence provide clues regarding the biochemical mechanisms involved in the origin of the teratological syndromes with which we are concerned. Irrespective of the teratogenic compound used, the most effective protection discovered against the genesis of rumplessness is provided by supplementary pyruvate, and the most effective protection against the formation of the syndrome involving facial and appendicular skeleton during the later susceptible period is nicotinamide. These findings as well as considerable additional evidence, such as the effects on blood sugar and the results obtained with inhibitors of anaerobic glycolysis, seem to establish beyond doubt that all the malformations and syndromes of defects in this series of experiments are variations on a single theme, and that this theme concerns carbohydrate metabolism.

It has also been seen, however, that nicotinamide is not an equally successful supplement in all cases. With insulin, sulfanilamide, and eserine treatment, the symptoms of micromelia can be completely forestalled with proper amounts of nicotinamide. There is a fair presumption that in these instances diphosphopyridinenucleotide-mediated metabolic pathways are involved in the origin of malformations. In contrast, with boric acid treatment, there are strong indications that interference with riboflavin plays a causative role. From this evidence, and since none pointing in other directions has yet come to light, it would seem probable that all the teratogenic substances used by us during the later sensitive stages achieve their results by an interference with processes of dehydrogenation. The fact that nicotinamide and riboflavin are essential in the prosthetic groups of a large number of dehydrogenases, each of which catalyzes the dehydrogenation of a particular substrate, may provide a clue for the specific interactions occurring between the various teratogenic compounds and particular parts of the embryo. The individual reactions of parts may also, however, be a consequence of the specific uses to which, at the time of interference, these differentiating parts apply their energy resources.

It is clear, at any rate, from the evidence at hand that specific relations exist between certain teratogenic compounds and particular skeletal parts. For proof of this, it should be recalled that within the axis of long bones, irrespective of the time of treatment, insulin and sulfanilamide produce maximal changes in the tibiotarsus, boric acid and eserine in the tarsometatarsus; or, that within the foot skeleton, and again independent of time, boric acid has a preferential effect on the fourth and pilocarpine on the first toe. The chemical structure of compounds also plays a highly specific role in deciding whether or not a substance will be teratogenic at all; thus, it is known that reversibly inactivated insulin becomes harmless in proportion to the degree of inactivation (Landauer and Lang, '46) and that isocolchicine has none of the teratogenic qualities of colchicine (Ancel, '52).

Attention should be called to certain additional features of our data. In the insulin experiments it was seen that stage differences of treatment led (between 3.5 and 7 days) to the same end effect, but that changes in dosage during one and the same stage produced differing end results. These observations seem to indicate that the teratogenic substances do not create continuing dissimilarities of developmental rate, but that they produce a time-limited change with subsequent compound-interest accumulations of effect according to local needs at the moment of interference. We recall the short span of time during which, after treatment, protective supplements remain effective. These facts point to local rather than systemic changes. At the same time it is also clear that the effect of the teratogenic substances cannot simply be one of destroying or rendering unavailable nutrient substances present in the yolk, else there should not be the relatively sharp changes at the beginning of sensitive periods. We are thus led to the assumption that the primary effect of our treatments was a temporary blockage of the supply of certain coenzymes with secondary differential effects on the embryo. In this respect, as in the morphological aspects, the chemically induced syndromes resemble many of those arising as a consequence of vitamin B deficiencies.

All the teratogenic compounds used in our experiments, if applied in sufficient amounts, produce an effect on general body size of the embryo. This may be attributable to the need of dividing cells of the chicken embryo for access to carbohydrate energy sources (O'Connor, '52). At the same time, it follows from our own observations and those of others (e.g., Spratt, '52) that, because of differential needs, there are differential responses of organs and parts to extrinsic factors disturbing metabolic pathways. Parts of the cartilaginous skeleton and neural crest cells appear to be most sensitive to the conditions imposed by our experiments. Secondary defects, such as cleft palate, presumably are the result of asynchronies of developmental rhythms of adjoining parts.

Finally, our observations must be considered in terms of the phenocopy concept. It seems certain that the teratological syndromes produced in our experiments and the features of the several mutants copied by them have more in common than superficial and coincidental similarity of terminal expression. Yet, there are also, I believe, strong arguments in favor of fundamental dissimilarities in the epigenetic mechanisms.

All our knowledge about mutants points to the conclusion that alterations of the normal phenotype are due to changes in or complete suppression of the elaboration of specific enzymes and, more specifically, that the protein part of these enzymes (apoenzymes) is involved. There is no reason to assume that mutations of fowl, such as the Creeper, Cornish and. ''short upper-beak'' lethals, are exceptions in this respect. In the case of our experimental syndromes, on the other hand, we have definite reasons to conclude that interference with cofactors rather than with enzymes is responsible for the deviations from normal morphogenesis. A similar situation has been encountered by Hadorn and his associates (Hadorn, '51) in comparative studies of *Drosophila* mutants and their phenocopies. Like these investigators, we are led to the inference that the complex chain of events which leads to the normal phenotypic traits is, in the two instances, broken in different places. The results of our experiments, similar to those on *Drosophila,* are probably mediated by differential starvation in essential metabolic factors, but there appears to be a much higher degree of specificity in the production of these phenocopies than was assumed by Hadorn for his material.

This interpretation of our observations helps to explain the similarities as well as the discrepancies in behavior of our mutants and their phenocopies. Tests on three mutant stocks (Creeper, dominant and recessive rumplessness) with supplements protecting against the production of phenocopies (sodium pyruvate and nicotinamide) have so far given entirely negative results. Neither absence nor abnormality of enzyme activity can be overcome by supplying constituents of coenzymes. By the accumulation of modifying genes, on the other hand, such as we observed in our Creeper and selected ''short upper-beak'' stocks, alternative pathways may have been provided which lessen the impact of the mutant condition as well as the damage from teratogenic compounds.[3]

The sensitive periods of development are, in this view, simply those stages during which — either because of a pre-existing gene substitution or on account of imposed experimental conditions — critical needs of certain organs or parts become limiting and, if not satisfied, produce gross alterations of normal morphogenesis.

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[3] It is interesting in this connection that in work with *Drosophila* Goldschmidt ('38) referred to the additive action of genetic and phenocopic agencies in the production of scalloped wings as an important contribution for proof that ''the processes underlying the formation of phenocopies are the same as those set in motion by mutant genes.'' This can be true, of course, but our evidence demonstrates that the opposite situation may prevail as well.

It remains an important fact, however, that the chemical methods of phenocopy production may furnish valuable clues about the nature of the metabolic pathways which are disturbed by mutant genes. One such clue, which I believe to be promising, is provided by our observations concerning the parallel effects of teratogenic compounds on cartilaginous skeleton and pigmentation. In two mutants of mice, the grey-lethal and microphthalmia, skeletal defects produced by disturbed bone absorption are associated with deficiencies of pigmentation, and in the grey-lethal, in addition, hypoglycemia tends to occur (Grüneberg, '35, '48; Hertwig, '42; Watchorn, '38). These seemingly obscure associations, for which no reasonable "pedigree of causes" was heretofore available, may find their explanation in the similarity of sensitiveness which mesenchymal and neural crest derivatives have shown under the conditions of our experiments, a similarity of reaction which presumably can be traced to related needs for and interferences with specific sources of carbohydrate energy, but which may in some instances also trace to relatedness of cellular origin.

It is clear that, as far as developmental genetics is concerned, the experimental production of phenocopies can only provide first approximations. The phenocopy method is no substitute for biochemical or biophysical analysis. If, with Sherrington ('51), we must be mindful that "to pick out this or that particular contributory condition in a conspiracy of reaction and to label that particular one the 'cause' is arbitrary and artificial," we yet may be able to derive from experimental work such as has here been reviewed certain significant generalizations.

Three generalizations are, I believe, justified from our present evidence: (1) Modifications of development with morphologically similar end effects can be produced by chemically specific interference at different points along one and the same pathway of metabolic functions. (2) The occurrence of syndromes of malformations involving several independent parts and organs of the embryo finds its explanation in relatedness of metabolic requirements during particular sensitive stages of development. (3) The developmental defects which have been discussed — presumably those of genetic as well as those of experimental origin — are brought about by an intervention in metabolic events on the cellular level. One may hope with confidence that continued experimentation in this field will in time lead to further broadening of the ground common to embryology and genetics.

DISCUSSION

A Voice: Since sulfanilamide is an inhibitor of carbonic anhydrase, have you tried any of the newer inhibitors of this substance?

Landauer: No. Ancel compared the effects of various sulfa compounds — *N*-sulfanilylacetamide produces results similar to sulfanilamide; others (soluseptacine, sulfapyridine, pulmorex, neoprontosil) are less effective. The complete protection against the teratogenic action of sulfanilamide afforded the embryo by nicotinamide, in proper amounts, would seem to exclude inhibition of carbonic anhydrase as a major factor.

Chairman Dunn: One feature of the new material reported which seems to deserve special comment is the correlation between the pigment restriction and the degree of morphological abnormality. If that is a new pathway through which these teratogenic effects are mediated, then there is a kind of morphological intermediary between some of the biochemical pathways which have gone awry, and so much is known about the origin, distribution, and fate of the melanophores that this would seem to offer a very promising new avenue of correlating morphological events on the lower level, as on the cellular level, with the biochemical ones that preceded.

In that respect I should like to ask if the correlated pigmentary restriction and morphological effects occurred at the early part of the sensitive period to which you referred, or at the later part.

LANDAUER: The later part. Our work with black embryos in early stages has not been very extensive but as far as it has gone, no effects on the pigment have been noted after treatment at either zero or 24 hours. Injection at either 96 or 120 hours produces the association of these two effects. And, as you said, there is a correlation between the degree of lack of pigmentation and the degree of morphological abnormality.

Chairman DUNN: That means, then, that the pigmentary effect is correlated with morphological abnormalities only in those periods which fall within the sensitive period of, say, the limb.

LANDAUER: That is right.

Chairman DUNN: So there is another specificity indicated. Consequently, one perhaps could not use the most obvious assumption — that these cells are themselves contributing to the morphological structures which are deficient — since the correlation holds only with certain abnormalities.

LANDAUER: Presumably, the affected cells are related in their metabolic needs rather than involved in identical morphogenetic processes.

WAELSCH: Do you have any idea whether migration of the neural crest cells is disturbed or whether it is the actual production of melanin?

LANDAUER: No, we have no evidence as yet. I do know that abnormal white down feathers are replaced by black feathers, usually in the first but sometimes in the second molt after hatching. The experimental effect is always an extension of the normal condition, that is, there is no spotting but always a widening of the ventral white zone.

WAELSCH: Is it an interference with melanoblast migration?

LANDAUER: I think the distance the neural crest cells migrate from their point of origin is involved. There may be a selective mortality of neural crest cells along their path of migration, fewer arriving in distant areas, or there may be an interference with their activity.

WILLIER: In newly hatched Barred Plymouth Rock chicks, where the down feathers of the wing tips and undersurface of the body are characteristically white, the evidence based on an embryological study (Watterson, '42) indicates that, although the pigment cells migrate from the neural crest ultimately to the feather germs in these positions, either sufficient numbers of them fail to arrive in time or their entry into the epidermis of the developing down feathers is blocked in some manner. Whatever the explanation, it is clear that, immediately with the onset of the formation of the juvenile feather which succeeds the white down feather, the pigment cells appear abruptly in the epidermis and deposit black pigment in all feather parts in process of formation. The same kinds of phenomena are probably characteristic of the black Minorca fowl.

HICKS: We have given many drugs to pregnant and newborn animals and often find it necessary to press the agent to the sublethal level to get an effect. Some agents — many, in fact — exhibit no selective action short of killing the animal outright. I should like to know what dosages you used in your experiments.

LANDAUER: Most of the dosages are very high. Injection of 3 mg of pilocarpine at 96 hours permits almost normal hatching, about 16% of the chicks being syndactylous but otherwise normal. Six milligrams reduces the hatch to less than 40%. A dose of 12 mg prevents hatching; most of these chicks survive to late stages but all die before hatching. Increasing dosages inevitably increase the incidence of extreme abnormalities. Boric acid injections present a very narrow experimental range. The results presented here were from 2.5-mg injections. Practically all embryos given 3.5 mg are killed immediately while dosages a little below 2.5 mg are quite ineffective.

Chairman DUNN: Dr. Landauer's discussion seems, actually, to have brought to a crux some of the essential questions which this conference has been considering, i.e., those having to do with the interaction between specific genetic constitutions and specific environmental agencies, or the relation of physiological genetics to experimental teratology. As someone said we come in somewhat more refined terms to the question of heredity and environment, and are required now to specify much more carefully than was ever anticipated when this kind of study began, the specific ways in which the differentiated genotypes or the species respond to the varieties of environments in which they find themselves.

Dr. Landauer chose to put the chief weight of interpretation on the alteration of specific biochemical pathways, specifically, on enzyme processes intervening between altered genes and altered phenotypes.

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PHASES IN EMBRYONIC DEVELOPMENT[1]

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As a basis for gaining some insight into the way that radiation and chemical agents influence the developmental processes at particular times and places, it is essential to bear in mind that the formation of the embryo from the fertilized egg involves a series of successive and interconnected events, each of which is dependent on preceding events. Although development is a continuum and is progressive, it is possible to characterize arbitrarily the main features peculiar to the different stages or periods. For purposes of illustration, a time scale in the development of the chick embryo will be used. On this time scale an attempt will be made to characterize the main phase in sequence from a period prior to laying of the egg to the time of hatching.

Phase of morphogenesis. The most striking feature of the initial period in development is morphogenesis, i.e., those modeling changes that bring about the specific form characteristic of the organism — or, to put it in another way — those processes concerned with the origin of specific form. During the early part of this period the three germ layers are established in stepwise fashion by an orderly shifting or movement of cells or sheets of cells from one position to another (''morphogenetic movements''). The result is an orderly relation of one layer of cells to another. Through an interaction of the newly arranged layers, new features arise which are dependent on preceding events.

Any external agent that disturbs the normal interrelations of the primary germinal tissues would lead to a morphological disturbance of a distinctive kind and peculiar to this period in development. For example, a localized disturbance in the growth or physiology of the prechordal mesoderm, either prospective or actual, could lead to an abnormal development of the overlying neural ectoderm, resulting in a cyclopean eye. Although it is generally thought that the specificity of such an effect lies in a critical moment of development at a particular locus, the effective time of action of the deleterious agent cannot be very narrowly defined, since the first visible effect is undoubtedly preceded by a succession of events.

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[1] This modest work is respectfully dedicated to the distinguished embryologist, Professor F. Baltzer, in honor of his seventieth birthday.

Through the formative movements as outlined, the ground plan or framework of the future embryo is established. Definiteness of body form is foreshadowed, for such directional properties as the main head-tail axis and bilateral symmetry of the body are clearly discernible. Furthermore, within this initial framework of organization a number of organ-forming areas or fields have been detected by experimental means (Rudnick, '32; Willier and Rawles, '35; Clarke, '36; Rawles, '43). Each area, such as that of the eye, heart, kidney, and thyroid, occupies a definite spatial position in relation to the other areas and to the whole blastoderm. Three points of special interest stand out: (1) Each organ-forming area exhibits a gradient in histogenetic potency, the intensity of which is high in the center, gradually diminishing peripherally until it completely disappears. Furthermore, each area has far greater potencies for a particular organ than can possibly be the case in normal development. (2) Areas lying across the median axis, e.g., the eye, exhibit a definite asymmetrical organization, the left portion showing a far greater histogenetic potency than the right, and the median portion the highest of all. (3) With advance in development a progressive restriction in histogenetic capacities of the various areas apparently takes place, with the result that, just prior to the formation of a given organ primordium, the corresponding local area is limited in its potentialities to the organ-forming system in question.

Phase of organogenesis. Within this basic spatial pattern a further series of morphogenetic or form changes, such as budding, folding, and differential growth, takes place. These culminate in the formation of a primordium (anlage or rudiment), the earliest discernible stage in the formation of an organ. Each organ primordium arises in a definite spatial position and in an orderly sequence within the original framework. Moreover, each primordium behaves to a high degree as a morphological unit, exhibiting changes in shape, in histogenesis, and in growth pattern peculiar to it. Although limited in direction of differentiation, each primordium exhibits the properties of an embryonic system in that a series of successive and interconnected events proceeds in an orderly manner within it. Such an organ-developing system undoubtedly possesses critical moments in its realization.

A localized disturbance in a developing primordium (or its precursor material) may disrupt its subsequent development and, as a consequence, it develops abnormally. For example, the removal of a small piece of skin ectoderm from the tip, but from no other locus, of an outgrowing limb bud of a chick embryo inhibits completely the further formation of the parts of the limb (Saunders, '48). The tip ectoderm is the critical tissue component for the sequential development of the limb parts (skeleton and muscles) in a proximal-distal order.

Phase of initial functional activity. The series of changes in morphogenesis and histogenesis of an organ primordium soon culminate in the acquisition of properties for specific physiological function. That is, with the elaboration of form and appropriate structures, each organ reaches a point or stage in organogenesis when it begins to display signs of functional activity. For example, as may be seen in table 1, the liver begins to accumulate glycogen on the sixth day, and the thyroid begins to store thyroglobulin in follicles on the ninth day. In sum, the onset of function is the resultant of a multiplicity of events which proceed in a stepwise manner and, in a measure, independently of a direct rapid exchange of chemical substances among the developing organs. A change in the timing of any one step or process in the chain of events leading up to functional activity may disrupt the sequence at any step, with the consequence that subsequent steps proceed in a modified or abnormal fashion or cease altogether. The end result may be either the lack of or the abnormal function of the organ. Furthermore, disturbances in the establishment of proper functional connections of nerves and blood vessels with the developing organ could conceivably disrupt or alter the processes of organogenesis and lead to functional disarrangement.

Phase of functional integration. Once the initial functional phase of an organ is attained, signs of increased functional activity with advance in developmental age become clearly apparent. For example, as is seen in table 1, the quantity of liver glycogen in the normal chick embryo gradually increases from a trace on the sixth day to a value of 3.90% (dry weight) on the tenth day and, save for a temporary drop at the twelfth day, continues to rise to its highest value shortly before hatching. Of special note here, however, is that the period from about the tenth through the thirteenth day is one in which the humoral communications between organs via the blood-vascular system seem to make their appearance. Seemingly, the organs are no longer independently functionally active, but are interconnected in their functional activity. Evidence in support of this concept is brought together in summary form in table 1, an inspection of which will show a variety of events which are peculiar to the 10- to 13-day period. The main points of significance are briefly summarized as follows:

1. Onset of active histogenesis of the cell types in the anterior pituitary.
2. Onset of synthesis and storage of thyroglobulin.
3. Onset of hormonal regulation by the anterior pituitary, since its removal suppresses the synthesis and storage of thyroglobulin and the accumulation of lipids and ascorbic acid in the adrenal cortex.
4. Retrogression of the incipient ovarian cortex of left testis and of oviducts in normal genetic males completed. Early injection of estradiol prevents their retrogression.

TABLE 1

Summary of correlations in development of humoral activity

ORGAN OR TISSUE [a]		TIME (DAYS) 6	8	9	10	11	12	13	14	16	18	20	COMMENTS	AUTHORITY
Anterior pituitary	Acidophiles				+	++		+++			+++++			Payne, '46 Rahn, '39 Wilson, '52
	Basophiles	Immature										++	Definitive after hatching	
Thyroid	Follicles + colloid (N)			+		++		+++			+++++		Increase in number, size, and amount of colloid	Fugo, '40 Hopkins, '35 Martindale, '41
	Follicles + colloid (–P)			+		+		+		+	+		Relatively few, small size; little or no colloid	
Adrenal cortex	Ascorbic acid (N)	?					0.95					1.67	Values in $\mu g/mg$	Case, '52
	Ascorbic acid (–P)	?					0.93			0.99		1.10	Values in $\mu g/mg$. Tends to remain constant at 12-day level	
	Lipids (N)	?					+++				+++++			
	Lipids (–P)	?					++				++		Little or no apparent increase: ACTH restores to normal	
Testis (left)	Ovarian cortex (N)	———	———	———	———	———							Complete disappearance by eleventh day	Willier *et al.*, '37 Willier, '39
	Ovarian cortex (estradiol)	———	———	———	———	———	———	———	———	———	———	——→	Injection prior to eighth day: no effect after eleventh day	
	Oviducts (N)	———	———	———	———	———							Complete disappearance by eleventh day	
Oviducts	Oviducts	———	———	———	———	———	———	———	———	———	———	——→	Injection prior to eighth	

5. Carbonic anhydrase, gamma globulin, and both constituents (fibrinogen and prothrombin) essential for blood clotting first detectable in the blood.
6. The occurrence of a "plateau" in blood sugar level associated with a temporary drop in the amount of liver glycogen. It is not without significance that the pancreatic islets for the first time show β cells with secretion granules, the commonly accepted cellular source of insulin.
7. Changes in quantities of blood sugar and liver glycogen after early pituitary removal. Although inexplicable on the basis of available evidence, these changes may be attributed tentatively to some disturbance in the hormonal balance.
8. In connection with items 5 and 6 it is of significance to note that, after injection of insulin (5 units) into the yolk sac of a 5-day chick embryo, (1) the blood sugar is markedly reduced from the sixth to the twelfth day, rising to normal levels by the fourteenth day (Zwilling, '48), and (2) all carbohydrate fractions are diminished from the sixth to the twelfth day, but free sugar and glycogen tend to return to normal levels by the twelfth day of incubation (Zwilling, '51).
9. A final point of interest (not shown in table 1) is that a sharp increase in actomyosin of skeletal muscle occurs, beginning on the twelfth day (Herrmann, '52).

Correlations in time of such a variety and multiplicity of events as have been summarized and listed in table 1 (the listing is still incomplete) are indeed very striking during this period. The many glands and organs of the embryo apparently have become correlated functionally with each other in a chain-like manner via the blood-vascular circulation. In all probability, the action of one gland regulates the activity of another, which in turn may have a reciprocal effect such as to accelerate or suppress its activity. Every link in the functional chain must fit together in a specific way for the proper functioning of the embryo.

	anhydrase (N)					eighteenth day (adult level)	Clark, '51 Van Goor, '40
Blood	Fibrinogen + prothrombin (N)			+ ——	——	Always present by fourteenth day	Pickering and Gladstone, '25
	Gamma globulin			+ ——	——		Nace, '53
	Sugar (N)	87	112	112	151	Values in mg %; increasing to peak after hatching	Konigsberg, '52 Zwilling, '48
	Sugar (–P)	119		181	150	Values in mg %; hyperglycemia → hypoglycemia	Konigsberg, '52
Liver	Glycogen (N)	Trace	3.90	2.32	7.17	Values in percentage of dry weight rises to highest value on nineteenth day; drops at hatching	Konigsberg, '52 Leibson, '50 Zwilling, '51
	Glycogen (–P)		3.90	8.35	9.40	Values in percentage of dry weight	Konigsberg, '52
		Independent functional development	Onset of chemical integration		Increasing functional interdependence		
		State of chemical coordination					

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[a] N = Normal embryos.
–P = Embryos lacking the pituitary.

On the basis of such a complexity of coordinating mechanisms, it is clearly evident that, irrespective of how it is produced, a functional disturbance in one gland can influence the functional activity of another gland even though its capacity for functioning is normal. In a similar way it is apparent that disturbances in one or more humoral constituents in the blood circulation could lead to an imbalance, thereby setting up a chain of atypical processes. The terminal effects of such disturbances may be manifold in kind, depending not only on the substance missing or altered, but also on the specific reaction tendencies of the different tissues and organs. One tissue may be affected whereas another is not. The end effect may be displayed in the form of aberrant growth, function, or structure or the combination of the three.

Phase of functional maturation. The period from approximately the fourteenth day to the time of hatching may be regarded as a second phase in development of functional activity. It is characterized in the main by a progressive increase in amount of specific glandular tissue, in quantity of glandular secretory products, and in quantities of certain chemical constituents of the blood. It would appear that the functional interlocking of the glands, via the humoral substances, which is initiated at least in part during the 10- to 13-day period becomes strengthened and extended during the latter third of embryonic life; in all probability, however, it does not attain full expression until the postembryonic stages of development.

Conclusion. It is hoped that this brief characterization of the main phases in the time scale of the development of the chick embryo may have indicated at least the general nature of the sequential processes that may be influenced at a particular time and locus by radiation or chemical agents (see Hamilton, '52). Each major phase in development apparently represents a transition from one state to another, irrespective of whether the change is structural or functional in nature. Furthermore, each major phase seemingly comprises a series of less conspicuous steps, any one of which might be regarded as a critical or sensitive phase for the moment of action of the deleterious agents. The broad problem remaining for elucidation is to trace and to characterize more precisely than heretofore the sequence of processes that takes place from the initial time of action of the inducting agent to the terminal effect. The prevailing approaches to this problem have been largely confined to a description of the terminal effects, while the causal analysis of the underlying events has not yet been explored on a scale commensurate with its great importance. Indeed, the assertion of William Harvey made exactly 325 years ago, ". . . that all we know is still infinitely less than all that still remains unknown," is equally pertinent here (quote from dedication of treatise "An Anatomical Disquisition on the Motion of the Heart and Blood in Animals").

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A STUDY OF THE OUTCOME OF PREGNANCY IN WOMEN EXPOSED TO THE ATOMIC BOMB BLAST IN NAGASAKI

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THREE FIGURES

This study was conducted in Nagasaki during the first half of 1951. A list was made of all women who, at the time of the explosion, were of childbearing age (17 to 50 years of age on the basis of pregnancy records of the Atomic Bomb Casualty Commission Genetics program) and were residing in Nagasaki. From a total of 1774 women of childbearing age who survived within the 2000-meter area, only 98 women were found to have been pregnant on August 9, 1945. Conception was considered to have occurred 2 weeks after the last normal menstrual period. This group of irradiated mothers was further divided into (1) those mothers who had one or more ''major'' diagnostic signs of radiation injury, i.e., epilation, purpura and petechiae, and oropharyngeal lesions, and (2) those who had no evidence of radiation disease, or only ''minor'' signs.[2]

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[2] Of the symptoms commonly encountered in the syndrome of radiation sickness, epilation, purpura and petechiae, and oropharyngeal lesions, are generally considered to be the most reliable evidence of radiation injury and are designated in this study as ''major'' diagnostic signs. Malaise, vomiting, fever, and diarrhea, which are frequently manifestations of radiation sickness, may occur with many other illnesses. Considering the sanitary conditions which prevailed in the city immediately following the explosion, these latter symptoms are considered of ''minor'' importance in diagnosing radiation disease.

In selection of the control group, 4000–5000 meters was considered to be a distance sufficiently remote from the hypocenter so that only minor effects of the explosion occurred. In selection of the area from which the control group was drawn, due regard was given to the "fall-out" area where radioactive fission products from the bomb were said to have fallen.[3] In order to insure maximum comparability, selection of the control group was based on consideration of such factors as age distribution and similarity of population groups. In the control area, 1774 women of childbearing age were chosen at random, of whom 113 were found to have been pregnant on August 9, 1945.

Detailed pregnancy records and medical histories were obtained from the mothers in both groups. The questionnaire was designed to consider the traumatic, thermal, and radiation effects as to time of onset, duration, and severity. Information on shielding was obtained, but sufficiently detailed data for qualitative or quantitative evaluation could not be gathered. Serological tests for syphilis were performed whenever the history or findings indicated.

The following definitions were used to define fetal, neonatal, and infant mortality:

1. Abortion: The spontaneous expulsion of a conceptus at the twentieth week of gestation or earlier.
2. Stillbirth: Fetal death prior to the complete expulsion of the fetus from its mother after the twentieth week of gestation.
3. Neonatal death: Death occurring within 1 month after the birth of the infant.
4. Infant death: Death occurring after the first month and during the first year of life.
5. Child morbidity: This term is reserved in this study for children who were found to be mentally retarded on clinical examination.

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[3] Yamasaki, M.: Observations of the fall-out area in Nagasaki, 1951. Atomic Bomb Casualty Commission. Unpublished.

Detailed histories of pregnancy terminations were taken. For all postnatal deaths a postmortem history was obtained. Those children who had been fetuses *in utero* at the time of the bombing and were still residing in Nagasaki during the period of this investigation were seen at the Commission's clinic, where a careful clinical assessment was undertaken including anthropometric measurements, determination of the child's developmental level, hematological studies, bone age determination, examination and culture of the stool for ova and parasites, and serological test for syphilis.

Of the 98 mothers within 2000 meters, 77% of whom were within 1500 meters of the hypocenter, 30 developed one or more major signs of radiation effect. The remaining 68 mothers had no signs, or had signs of radiation not considered major. Trauma either to the extremities or the torso occurred in two-thirds of those with major signs and in one-third of those without the major signs. A comparative evaluation of the severity of the injuries was not possible. Within 2000 meters only 4 of the 98 mothers gave a history of severe flash burns, of which only one was extensive. The low incidence of burns among these mothers was undoubtedly a factor in determining their survival, since burns which occurred within 2000 meters were usually severe enough, when combined with radiation injuries, for death to ensue.[4]

The pregnancy termination among irradiated and control groups with reference to fetal, neonatal and infant mortality, and child morbidity rates is indicated in figure 1 and in tables 1, 2, and 3. Among the thirty mothers with one or more major signs of radiation, there was an over-all morbidity and mortality of approximately 60%. According to the stage of gestation, the over-all morbidity and mortality rates in the group with major signs were equally distributed in all trimesters, but in the group with no major signs only the first trimester pregnancies seemed to be similarly involved.

[4] Shirabe, Raisuke: Medical survey of atomic bomb casualties, 1951. Unpublished. (Professor of Surgery, Nagasaki Medical School)

Of the thirty pregnancies in the group with major signs, there were seventeen children who survived infancy, one of whom died of dysentery at 2 years of age. The following observations were made on the 16 remaining children: 12 were mentally normal, while 4 were found to have varying degrees of mental retardation. All four were noted to be relatively retarded in their physical development. The mothers of these

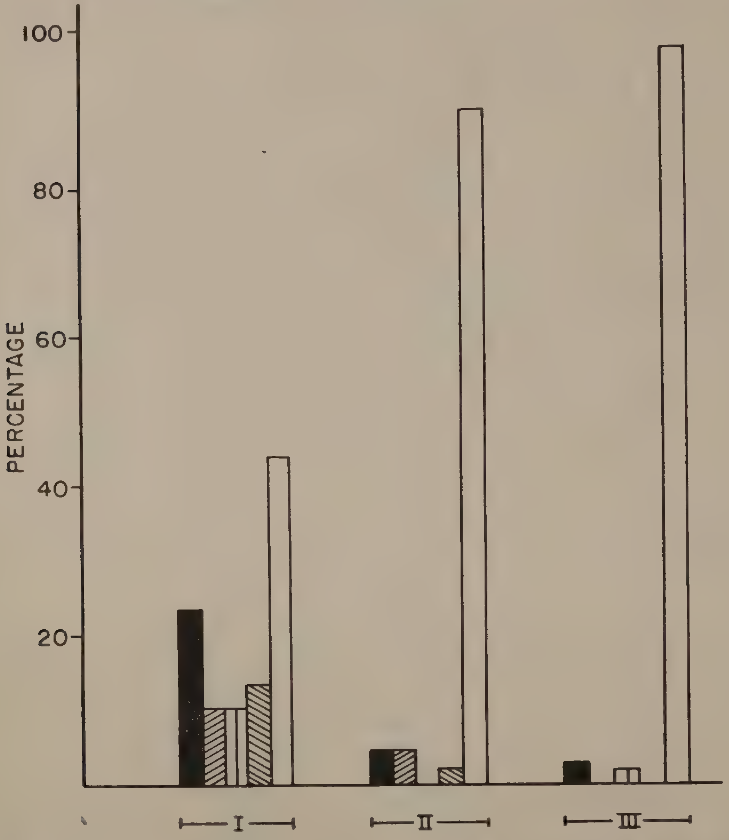

Fig. 1 Pregnancy outcome of women exposed to the atomic bomb blast—Nagasaki.

I. 0–2000 meters, with "major" radiation signs.
II. 0–2000 meters, no "major" radiation signs.
III. 4000–5000 meters, controls.

■ Fetal death; ▨ neonatal death; ▥ infant death; ▧ mental retardation; □ normal child.

children had been 850–1150 meters from the hypocenter. Moderate to severe impairment of speech was noted in these children. Two had never talked by the age of five; speech was incoherent in another; and in the fourth, speech was intelligible only to the immediate family.

In the irradiated group *without* major signs and the control group, the child morbidity rates were 1.6 and 0%, respectively.

Child morbidity according to the stage of gestation was higher in the irradiated group with major signs — 10, 20, and 10% for each trimester, as compared to the irradiated groups without major signs where the incidence was 8.3, 0, and 0% for the first, second, and third trimesters, respectively (table 2).

TABLE 1

Fetal mortality among irradiated and control groups

GROUP	NO. OF CONCEPTIONS	NO. OF ABORTIONS	NO. OF STILLBIRTHS	PERCENTAGE OF FETAL MORTALITY
0–2000 meters: ''Major'' radiation signs	30	3	4	23.3
0–2000 meters: No ''major'' radiation signs	68	1	2	4.4
4000–5000 meters: Controls	113	2	1	2.6

The mean height, weight, and head circumference of the children examined in clinic is shown in table 4. The mean height of the 16 children born to mothers with major radiation signs was 98.6. cm. The observed difference in mean height between the control and major radiation signs group is 2.9 cm, and the probability of observed difference being due to chance is less than 0.01.

The mean weight in the groups was as follows: 15 kg in the children whose mothers had major radiation signs and 16.8 kg in the children whose mothers had no major signs; the ratio of height to weight in each group, including the control group, remained unchanged.

TABLE 2

Fetal, neonatal, and infant mortality, and child morbidity rates according to stage of gestation among irradiated groups

TRIMESTER OF PREGNANCY	0–2000 METERS: "MAJOR" RADIATION SIGNS				0–2000 METERS: NO "MAJOR" RADIATION SIGNS			
	No. of conceptions	Percentage of fetal mortality	Percentage of neonatal and infant mortality	Percentage of child morbidity	No. of conceptions	Percentage of fetal mortality	Percentage of neonatal and infant mortality	Percentage of child morbidity
I	10	30	0	10	12	8.3	16.6	8.3
II	10	30	20	20	27	3.7	. . .	. .
III	10	10	40	10	29	3.4	3.4	. .
Totals	30				68			

In figure 2, the height and weight measurements are indicated for children born to mothers with and without major signs of irradiation. It can be seen that children born to mothers with major signs, represented by open circles on the figure, are definitely retarded in their growth and development. Three of the five lowest circles represent children who were mentally retarded.

TABLE 3

Neonatal and infant mortality among irradiated and control groups

GROUP	MOTHERS[a]	NEONATAL DEATHS	INFANT DEATHS	PERCENTAGE OF MORTALITY
0–2000 meters: ''Major'' radiation signs	23	3	3	26.1
0–2000 meters: No ''major'' radiation signs	65	3	0	4.6
4000–5000 meters: Controls	110	1	3	3.3

[a] Mothers of living infants: stillbirths and abortions excluded.

TABLE 4

Mean height, weight, and head circumference of all children examined

GROUP	NO. OF CHILDREN	MEAN HEIGHT	MEAN WEIGHT	MEAN HEAD CIRCUMFERENCE
		cm	*kg*	*cm*
0–2000 meters: ''Major'' radiation signs	16	98.6 ± 3.29	15.0	47.18 ± 2.08
0–2000 meters: No ''major'' radiation signs	57[a]	102.6 ± 4.28	16.8	49.50 ± 1.42
4000–5000 meters: Controls	96[b]	101.56 ± 4.64		49.03 ± 1.15

[a] Five of the 62 children did not receive anthropometric measurements.

[b] Eleven of the 107 children did not receive anthropometric measurements.

The mean head circumference of children born to mothers with major signs was 47.18 cm and for children of mothers without major signs 49.50 cm. The mean circumference in the control group of children was 49.03 cm. The observed difference between the group with major signs and the control group was 1.85 cm and the probability of observed difference being due to chance is less than 0.01.

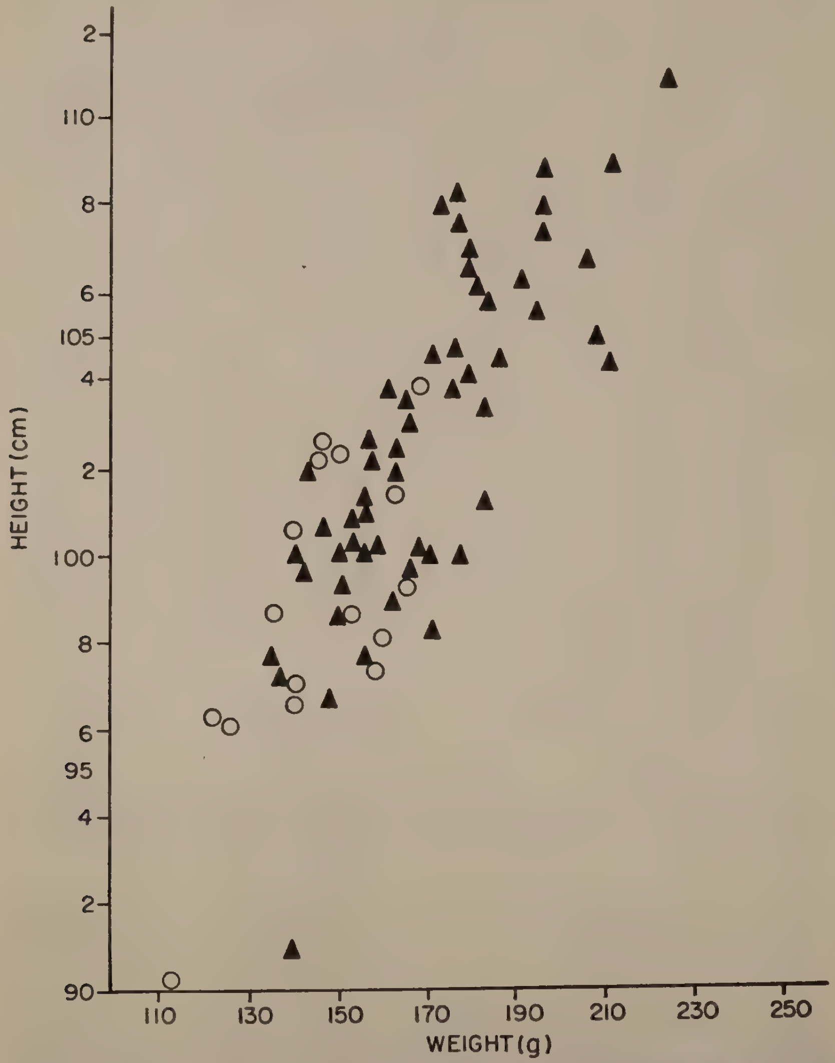

Fig. 2 Height and weight of children *in utero*, within 2000 meters of atomic bomb blast — Nagasaki. ○ Children of mothers with "major" radiation signs; ▲ children of mothers without "major" radiation signs.

The head circumferences of the three groups is shown graphically in figure 3. The head circumferences of the four children with mental retardation were 41.7, 44.3, 46.5, and 46.7 cm, respectively, accounting for the four smallest measurements in the group of children whose mothers had major radiation signs.

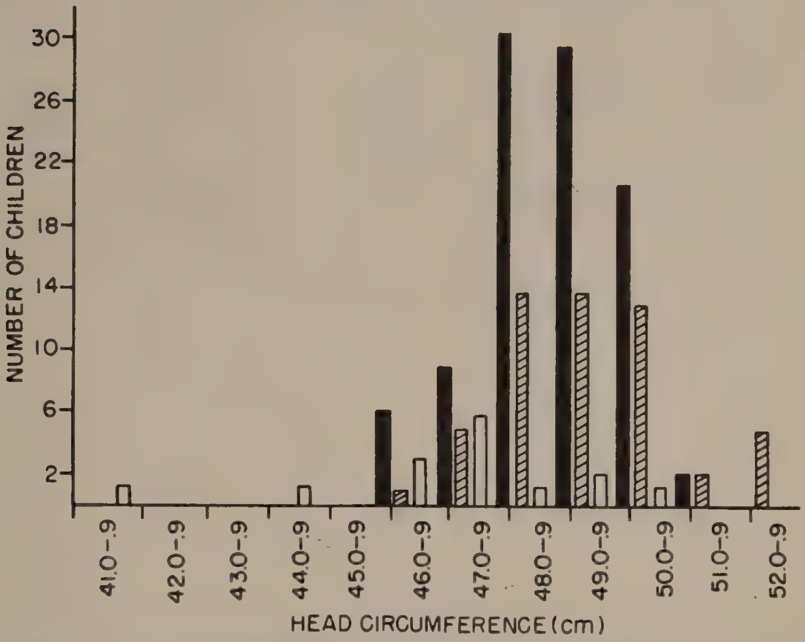

Fig. 3 Head circumference of children *in utero*, at Nagasaki, Spring 1951. ■ Mothers, 4000–5000 meters (controls); ▨ mothers without "major" radiation signs, 0–2000 meters; □ mothers with "major" radiation signs, 0–2000 meters.

SUMMARY

A study has been made of the outcome of pregnancy in women who were pregnant at the time of the atomic bomb explosion in Nagasaki. Among 30 mothers with one or more major signs of radiation, i.e., epilation, oropharyngeal lesions, purpura, or petechiae who were within 2000 meters of the hypocenter, there were 7 fetal deaths (23.4%), 6 neonatal and infant deaths (26%), and 4 instances of mental retardation among 16 surviving children (25%). The over-all morbidity and mortality is approximately 60% in this group of mothers. This is in sharp contrast to the group of mothers without major signs, but within 2000 meters where the over-all mortality was only 10%, and in the control group where it was about 6%.

The mean height and head circumference of children born to mothers with major signs was significantly smaller than in those children born to mothers in the control group.

It is difficult to evaluate the effect of radiation on this mortality and morbidity since other factors such as trauma, burns, infection, etc., may have a deleterious effect on the fetus. The evidence strongly suggests, however, that radiation either directly to the fetus, or indirectly as a result of its effect on the maternal tissues, was of considerable importance in determining the outcome of these pregnancies.

SUMMARIZING REMARKS

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If I may presume to talk to the non-embryologists and those who are not normally conversant with the developmental process, I shall address myself mainly to them.

In the first place, embryogenesis is not something which proceeds in a few discontinuous, nicely, neatly marked out phases. It is something which starts off with the whole plasma and the genetic system in it in a continuous series of transformations, a large number of separate chains of events in different parts of the organism, whose paths soon diverge but remain, as Dr. Willier has shown here, interrelated, in increasing complexity. If this is kept in mind, there will be no danger of misinterpreting such terms as ''critical phase'' or ''sensitive periods'' as if they were sharply demarcated phases. An objective view will be taken of the system at any one moment as it is at that time, with the idea of determining what a particular agent does at a particular time to a particular system, considering that the system prior to that time has already undergone a long series of transformations. The genes are no longer in the same setting of protoplasm that was present originally. The system has been transformed, and it must not be expected to be the same ever again.

It should be remembered that an agent may undergo a number of modifications in its course through the organism in the blood, or in the uterus if the object is a mammal; in the membranes if it is a chick; at the border of the cell, etc. It is not even known whether it gets into the cell. Therefore, it should be borne in mind that when different agents are employed they will neither reach the target all at the same moment, nor will all of them perhaps ever get to the target in the forms in which they were introduced.

In speaking of the reaction of the tissue, which is used as a final criterion, it should be remembered that this reaction consists of chains of physical-chemical processes in time, that is, kinetics. It takes time to get a tangible, recordable result; and that time, depending on the rate of the process, may be of the order of the second, the minute, the hour, the month, or the year. It is, therefore, impossible to tell from a particular external criterion or signal which has been chosen arbitrarily just when the action that later produces it set in. It is known only that the agent must have struck some time before its effect appears. The change is in the system, but its detection depends entirely on the observers, not on the system itself. Things which are properties of the human powers of observation, and their limitations, should not be projected back into the system. Finally, the developing organic system must not be considered as a microprecision clockwork in which any two individuals or any two systems can possibly ever behave in exactly and precisely the same way. This is what the geneticists describe as variability. The same thinking should be transferred to the group, to the population of cells being studied. They come from a common origin, but do not remain identical. They are subject to different accidents in their individual histories, and they eventually constitute a population, which in all respects, presumably conforms to a normal distribution curve. In such a cellular population, the ordinates being frequencies and the abscissas being thresholds of response, obviously, if an agent of a given dosage, intensity, or duration is applied all the cells up to a certain point on the abscissa are activated. An increase in the dosage will activate the cells up to the next point. It is the property of the integral of this normal curve that it is a nearly straight line for the median in about 85% of its course.

Therefore a linear increase with dosage of any such effect is really suggestive evidence of a random population of cells, where increasing numbers of singly dispersed cells are hit as the agent is increased.

If this were the case in radiation — it definitely can be shown in embryology — or with chemical agents, then the question arises: How can solid patches of cellular destruction — a multicellular mosaic — result from chemical or radiation agents?

Apparently, if an affected single cell in turn starts to emanate secondary effects around it, perhaps due to the release of chemicals from the necrosis of those primary target cells, those secondary effects will mark the spot, amplify it, so to speak.

Quite a number of the phenomena that have been described in the literature might be explained on this assumption. This type of thinking should be applied when dealing with tissues — statistical responses, secondarily modified by chain reactions, so to speak, of releases within the tissues.

Now with these few general comments, I shall return finally to some positive statements. Obviously, mitosis cannot be the sole factor affected by these agents. Whether chemical or radiation induced, their effect on synthesis of cytoplasm cannot be the sole trouble. Neither can the separation of the chromosomes be the sole defect, because some of these effects — radiation, for instance — do not show until very late, after a long lag period when all the cells which divide are well beyond the duration phase of the radiation.

Hence, perhaps for the time being, no single agent should be postulated, no royal road or key answer to the questions which have been posed. As can be seen, little is as yet known about the embryo, or about development; what really goes on inside is still shrouded in comparative mystery.

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